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MARKERS OF MALIGNANCY IN ADRENOCORTICAL TUMOURS

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Pennanen M, Raade M, Louhimo J, Sane T, Heiskanen I, Arola J, Haglund C: Adrenocortical tumors: High CT attenuation value correlates with eosinophilia but does not discriminate lipid-poor adenomas from malignancy. J Clin Pathol 66(12):1076–1080, 2013.

II Pennanen M, Heiskanen I, Sane T, Remes S, Mustonen H, Haglund C, Arola J: Helsinki score- a novel model for prediction of metastases in adrenocortical carcinomas. Hum Pathol 46(3):404–410, 2015.

III Pennanen M, Hagström J, Heiskanen I, Sane T, Mustonen H, Arola J, Haglund C: C-myc expression in adrenocortical tumours. J Clin Pathol 71(2):129–134, 2018.

IV Pennanen M, Tynninen O, Kytölä S, Ellonen P, Mustonen H, Heiskanen I, Haglund C, Arola J: IDH1 expression via the R132H mutation–specific antibody in adrenocortical neoplasias – prognostic impact in carcinomas. J Endocr Soc 4(4):bvaa018, 2020.

The publications are referred to in the text by their Roman numerals.

ABBREVIATIONS

ACA	adrenocortical adenoma
ACC	adrenocortical carcinoma
ACT	adrenocortical tumour
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
CI	confidence interval
CRH	corticotrophin-releasing hormone
CT	computed tomography
DAB	3,3'-diaminobenzidine
DX	dexamethasone
EDP	etoposide doxorubicin cisplatin
ENSAT	European Network for the Study of Adrenal Tumours
ESE	European Society of Endocrinology
HE	hematoxylin and eosin
HPA	hypothalamus–pituitary–adrenal
HPF	high-power field
HU	Hounsfield unit
HUCH	Helsinki University Central Hospital
IDH	isocitrate dehydrogenase
IGF-2	insulin-like growth factor 2
IHC	immunohistochemistry or immunohistochemical
IQR	interquartile range
MIB1	mindbomb E3 ubiquitin protein ligase 1 (antibody against Ki-67)
MRI	magnetic resonance imaging
NGS	next-generation sequencing
p27	protein 27, cyclin-dependent kinase inhibitor 1B
p53	protein 53
PI	proliferation index
ROC	receiving operating characteristic
SF1	steroidogenic factor 1
TMA	tissue microarray
UTR	untranslated region
WHO	World Health Organization

ABSTRACT

The characterisation of adrenal tumours has become an important clinical issue due to the widespread use of radiological imaging and, thus, the increased incidence of clinically unapparent lesions. Most primary tumours of the adrenal cortex are benign adenomas, whereas adrenocortical carcinomas (ACCs) remain rare and highly aggressive. The therapeutic strategy for ACCs differs from that for adenomas, making the accurate diagnosis of adrenocortical neoplasms imperative. The primary modality for the radiological assessment of adrenal tumours is unenhanced computed tomography (CT). A tumour with any malignant features and/or attenuation value >10 Hounsfield units (HUs) indicate indeterminance and require further examination.

Nonmetastatic tumours must be assessed histologically, identifying adverse features indicating malignant potential. The proliferation index (PI) has served as a supplemental tool in assessing the malignant potential of adrenocortical tumours.

The study cohorts consisted of consecutive adult patients with primary adrenocortical tumours operated on at Helsinki University Central Hospital (HUCH) between 2002 and 2008 (study 1) and between 1990 and 2003 (studies II–IV). Clinical data, tumour samples and appropriate unenhanced CT scans were collected. Tissue microarray (TMA) blocks were constructed for immunohistochemical (IHC) examinations.

The proportion of eosinophilic lipid-poor cells in adrenocortical tumours correlated with the CT attenuation value by HUs. The attenuation value cannot distinguish between lipid-poor adenomas and carcinomas. All carcinomas had attenuation values >21 HUs.

The Helsinki score is a histological and immunohistochemical score developed to predict the metastatic potential of adrenocortical tumours in adults. Furthermore, the Helsinki score uses three individual parameters—mitotic frequency, the presence of necrosis and the proliferation of the tumour—to define a carcinoma. Calculation [3 x mitotic activity >5/50 hpf + 5 x presence of necrosis + proliferation %] yields a score, whereby a cutoff value ≥8.5 diagnoses a carcinoma. The Helsinki score is a reliable and powerful diagnostic and prognostic system.

Transcription factor c-myc was shown to express in adrenocortical tumours. Benign adenomas show a prominent nuclear c-myc expression comparable to that of normal adrenocortical cells, whereas carcinomas show an increased cytoplasmic expression. Furthermore, strong cytoplasmic and weak nuclear c-myc expressions associate with a malignancy and an adverse outcome.

Positive mutation–specific isocitrate dehydrogenase 1 (IDH1) R132H immunohistochemical (IHC) staining correlates with a better prognosis among ACC patients. However, IDH1 R132H IHC does not distinguish between local and metastasised tumours. Using a targeted next-generation sequencing (NGS) panel and exon sequencing, no mutations to IDH1 could be found

In conclusion, in the preliminary evaluation of an adrenal tumour, the threshold for an indeterminate tumour can be increased from 10 to 20 HUs, at least amongst patients with no history of malignancy, since no carcinomas had attenuation values ≤21 HUs.

The Helsinki score accurately predicts the metastatic potential of adrenocortical tumours and the prognosis of carcinoma patients, outperforming the diagnostic and prognostic power of previous systems.

Since strong cytoplasmic and weak nuclear c-myc expressions associated with malignancy and shorter survival, c-myc IHC can serve as a prognostic marker in adrenocortical tumours.

Amongst ACCs, IDH1 R132H immunopositivity correlated with a better prognosis, although immunopositivity does not seem to associate with mutations to the IDH1 gene.

1 INTRODUCTION

Adrenal glands form part of the body's endocrine system, situated above the kidneys, and consisting of two functionally distinct parts: the cortex and the medulla. The adrenal cortex secretes corticosteroids and androgens, whilst the medulla secretes catecholamines.

Adrenal cortical nodules are a common finding, reaching an overall incidence of 10%. Because some nodules are hyperplastic, the incidence of true adrenocortical neoplasias remains unknown. However, incidence has recently increased, due to the widespread use of radiological imaging, increasing the number of incidentally discovered adrenal lesions, or 'incidentalomas'. Furthermore, the ageing population in the Western world contributes to this increasing incidence, since incidence typically increases with age.

Most adrenal tumours are benign cortical adenomas detected either through symptoms caused by excess hormone secretion or as incidental findings in radiological imaging. Hormonally active adenomas primarily secrete aldosterone, although less frequently they secrete cortisol and very rarely sex steroids. Adrenocortical adenomas (ACAs) primarily affect older populations, impacting both sexes equally. No environmental predisposing factors have been associated with ACAs.

Whilst rare, adrenocortical carcinomas (ACCs) feature a stable annual incidence of 1 to 2 cases per 1 million population. The age distribution has two peaks. Most cases occur during the fifth decade of life, with another peak including children suffering from hereditary tumour syndromes. ACCs are found in women more often than in men, at a ratio of 2.5:1. Most adrenocortical neoplasias are sporadic, arising from random mutations of genes involved in the cell's signaling pathways. However, a slightly increased risk for ACC has been linked to smoking. Both adenomas and carcinomas can be associated with hereditary syndromes. The most significant syndrome is Li–Fraumeni, which causes 50% to 80% of paediatric ACCs. Although carcinomas are primarily detected based on symptoms caused by excess hormones or mass effect, incidentalomas may also prove to be carcinomas (Lloyd et al. 2017a).

Evaluating adrenal tumours includes a patient history, clinical examination, biochemical measurements and radiological imaging. Hormonally active tumours are normally operated on to cure the hormone-related syndromes they cause. Similarly, any tumours with malignant features must undergo surgery. Amongst patients with a history of malignancy, the possibility of metastasis to the adrenal gland must be considered. Small, inactive tumours with no malignant features do not necessitate operative treatment.

For adenomas, surgical resection represents a curative treatment. However, ACC is an aggressive disease carrying a poor prognosis. Thus, ACC patients require extensive imaging examinations to rule out metastases, adjuvant therapy to prevent recurrence and rigorous follow-up to detect the spread of disease as early as possible. This difference renders the accurate diagnosis of adrenocortical neoplasms imperative (Fassnacht et al. 2018).

Metastasis is a definitive sign of malignancy in adrenocortical tumours (ACTs) and the primary cause of a fatal outcome. In nonmetastatic tumours, the malignant potential must be assessed histologically. Since many histological parameters associate with malignancy, various histological scoring systems combining multiple parameters have been proposed to predict malignancy as accurately as possible. The most widely used system is the Weiss scoring system, which includes nine histopathological criteria representing adverse features of the tumour, whereby the presence of any three of these indicates a malignant potential. The proliferation index (PI) has served as a supplemental tool in assessing the malignant potential of ACTs. Moreover, immunohistochemical (IHC) markers and genetic classifications have been proposed to serve diagnostic and prognostic purposes (Lloyd et al. 2017a). However, none of the histological or molecular classification systems have achieved sufficient accuracy in predicting metastases.

2 REVIEW OF THE LITERATURE

2.1. Normal adrenal gland

Adrenal glands are yellowish triangular structures situated bilaterally above the kidneys embedded in the perirenal fat. The adrenal gland consists of two distinct parts, the function and embryological origin of which differ.

2.1.1. Development

The adrenal cortex derives from the mesoderm and secretes corticosteroids and androgens. The adrenal medulla originates from the neural crest and secretes catecholamines. During the embryonic period, the intermediate mesoderm of the urogenital ridge, together with gonadal primordial cells, gives rise to the adrenogonadal primordium at four weeks' gestation. The adrenal primordium then separates from the gonadal primordium. Cells with a neuroectodermal origin migrate from the neural crest via the sympathetic ganglion to the developing gland. A distinct, encapsulated foetal adrenal gland forms by embryonic week 9. The foetal adrenal cortex consists of a predominant foetal zone and a smaller surrounding definitive zone. The medullary cells are scattered throughout the foetal zone. Shortly after birth, the foetal zone rapidly involutes, the medullary cells aggregate and the definite zone grows (Schoenwolf et al. 2015).

2.1.2. Anatomy

The adrenal gland is enveloped by a thick, connective-tissue capsule, from which fibrous trabeculae with blood vessels and nerves extend to the parenchyma. The blood supply to the adrenal gland enters from three arteries: the superior, middle and inferior suprarenal arteries. These arteries branch before entering the capsule and give rise to three kinds of vessels: 1. capsular capillaries that supply the capsule; 2. cortical sinusoidal capillaries that supply the cortex and carry blood to the medullary capillary sinusoids; and 3. medullary arterioles that pass through the cortex along the fibrous trabeculae and supply the medullary capillary sinusoids. Thus, the medulla has a dual blood supply. This vascular system allows cortical hormones to influence the activity of the medulla. Venous blood is conveyed from the sinusoids to the adrenomedullary connecting veins that coalesce to the central adrenomedullary vein and drain into the inferior vena cava on the right side and the left renal vein on the left side. The capsule and the medulla contain lymphatic vasculature, but

not the cortex. The medulla receives abundant innervation from the T10 to the L1 spinal cord segments via myelinated presynaptic sympathetic fibres (Moore et al. 2014; Ross et al. 2011).

2.1.3. Histology and physiology

Cortex. The adrenal cortex is composed of three zones: the outer zona glomerulosa, the thick zona fasciculata in the middle and the inner zona reticularis (see Figure 1).

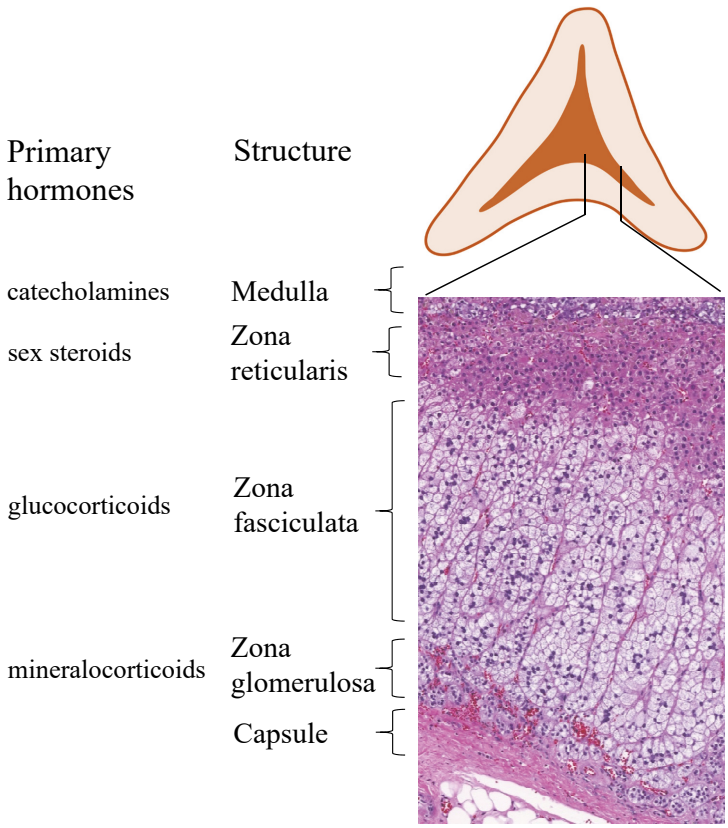

Figure 1. Histology of the adrenal gland and related hormones.

The cells of the zona glomerulosa are relatively small, forming clusters and columns. These cells produce mineralocorticoid aldosterone, which regulates salt and volume homeostasis. The zona glomerulosa is under the feedback control of the renin–angiotensin–aldosterone system. If the blood pressure or the blood sodium level decreases, the juxtaglomerular cells in the kidney secrete renin into the bloodstream.

Renin then catalyses the conversion of circulating angiotensinogen into angiotensin, which, in turn, stimulates the zona glomerulosa to secrete aldosterone.

The zona fasciculata consists of large cells in straight columns. The cells contain large droplets of lipids, the precursors for the glucocorticoids and sex steroids produced by these cells. The primary function of the glucocorticoids is to regulate glucose and fatty acid metabolism. They also depress the immune system. The zona fasciculata is regulated by the hypothalamus–pituitary–adrenal (HPA) axis (see Figure 2). The circadian rhythm and physical and mental stress stimulate the hypothalamus to release the corticotropin-releasing hormone (CRH), which then stimulates the release of the adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH stimulates the zona fasciculata cells to produce glucocorticoids. Both the secretion of CRH by the hypothalamus and the secretion of ACTH by the pituitary gland are, in turn, inhibited by glucocorticoids, creating a negative feedback loop.

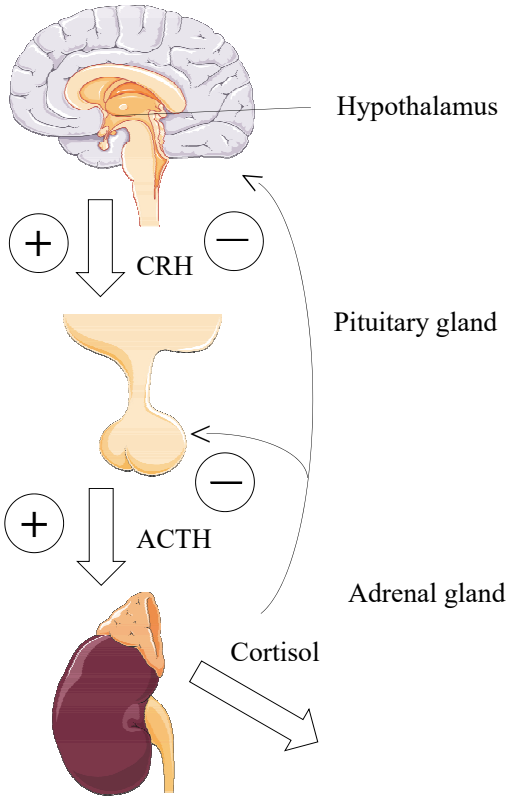

Figure 2. Infograph of the hypothalamic–pituitary–adrenal (HPA) axis. CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotropic hormone.

The cells of the zona reticularis are small and arranged in anastomosing cords. Some of the cells contain lipofuscin pigment granules. The zona reticularis primarily produces androgens and to a lesser extent glucocorticoids. Like the zona fasciculata, the zona reticularis is also regulated by the HPA axis (Ross et al. 2011; White et al. 2008).

Medulla. The parenchyma of the adrenal medulla consists of large pale-staining chromaffin cells that secrete the catecholamines adrenalin and noradrenalin in response to sympathetic nerve impulses. Chromaffin cells are similar to postsynaptic neurons, except that chromaffin cells secrete their products into the bloodstream. Catecholamines activate the body's flight-or-fight response. The medulla also regulates the secretory activity and blood flow of the adrenal cortex via ganglion cells (Ross et al. 2011; White et al. 2008).

2.2. Tumours of the adrenal gland

Tumours of the adrenal gland are categorised according to their tissue of origin. Table 1 summarises the World Health Organization (WHO) classification of tumours of the adrenal cortex and medulla.

Table 1. Classification of tumours of the adrenal gland according to the WHO (2017).

Adrenal cortex	Medulla
Adrenal cortical carcinoma	Phaeochromocytoma
Adrenal cortical adenoma	Neuroblastic tumours of the adrenal gland
Sex cord–stromal tumours	Neuroblastoma
Adenomatoid tumour	Ganglioneuroblastoma, nodular
Mesenchymal and stromal tumours	Ganglioneuroblastoma, intermixed
Myelolipoma	Ganglioneuroma
Schwannoma	Composite phaeochromocytoma
Hematolymphoid tumours	
Secondary tumours	

Adrenal cortical adenomas and carcinomas represent the primary tumours of the adrenal cortical epithelial cells. Other tumour types in the WHO classification originate from other cells and can appear in many organs of the body (Lloyd et al. 2017a). In addition to the primary tumours, secondary tumours appear in the adrenal glands, primarily in the setting of disseminated malignancies of different organs. The most common primary neoplasias metastasing to the adrenal glands are melanomas and breast and lung carcinomas (Angelousi et al. 2020).

Phaeochromocytoma is a primary tumour of differentiated chromaffin cells of the medulla. It is difficult to histologically distinguish between a benign and a malignant phaeochromocytoma. Only metastasis clearly defines a malignant phaeochromocytoma. Phaeochromocytomas affect people of all ages, although most present during the fourth and fifth decades of life. In addition, phaeochromocytoma is associated with a hereditary genetic susceptibility in about 30% of cases. Neuroblastic tumours of the medulla arise from more or less primitive cells of a neural crest origin and appear during childhood (Lloyd et al. 2017b).

In epidemiological settings, adrenal tumours are often grouped together, whilst reliable figures on adrenocortical carcinomas (ACCs) alone remain lacking. The Finnish Cancer Registry collects data from healthcare organisations on cancer cases in Finland. As shown in Figure 3, from 1957 to 2017, the incidence of adrenal cancer incresed in Finland. This increase in incidence results from advances in diagnostic methods across time.

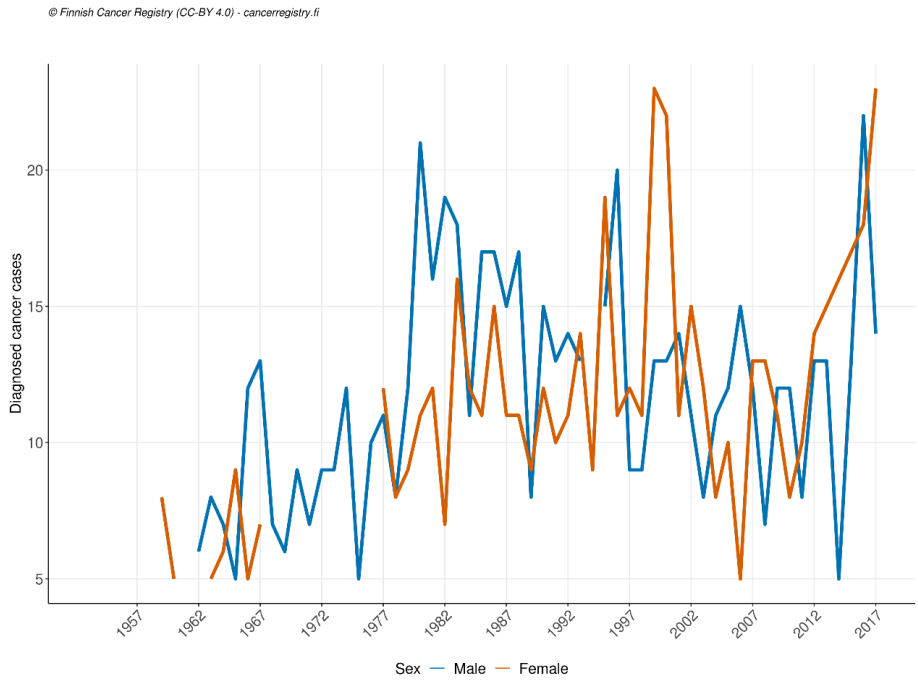

Figure 3. Incidence of adrenal cancer in Finland between 1957 and 2017. Data from the Finnish Cancer Registry.

2.3. Adrenocortical neoplasia

2.3.1. Epidemiology

Adrenal cortical nodules represent common findings, with an overall incidence of 10%. Because some nodules are hyperplastic, the incidence of true ACAs remains unknown. However, incidence has recently increased given the widespread use of radiological imaging, thereby increasing the number of incidentally discovered adrenal lesions (incidentalomas), primarily ACAs. In addition, the ageing population contributes to the rising incidence, since incidence increases with age. ACAs affect both sexes equally.

ACCs are rare, with a steady annual incidence of 0.5 to 2 cases per 1 million population. Southern Brazil emerges as a dismal geographical exception in incidence given the predisposing founder germline TP53 R337H mutation, where 0.3% of the population carry the mutation and 1 in 10 carriers are affected by ACC (Bugg et al. 1994; Figueiredo et al. 2006). The age distribution of ACCs features two peaks. Most cases occur near the fifth decade of life. Another peak occurs amongst children suffering from hereditary tumour syndromes. ACCs are found in women more often than men at a ratio of 2.5:1 (Lloyd et al. 2017a).

2.3.2. Aetiology

Most adrenocortical neoplasias are sporadic arising from random mutations of genes involved in the cell's signaling pathways. No environmental predisposing factors have been associated with ACAs. However, a slightly increased risk of ACCs has been linked to smoking. Both adenomas and carcinomas associate with hereditary syndromes (Lloyd et al. 2017a). The most significant syndrome is Li–Fraumeni, which causes 50% to 80% of paediatric ACCs. Other syndromes involved in the pathogenesis of ACCs, their mutated genes and prevalence amongst patients with ACCs appear in Table 2.

Table 2. Hereditary syndromes associated with adrenocortical carcinomas (Lloyd et al. 2017a)

Syndrome	Mutated gene(s)	Prevalence amongst patients with ACC
Li–Fraumeni syndrome	*TP53*	3–5% in adults 50–80% in children
Lynch syndrome	*MSH2, MSH6, MLH1, PMS2*	3% in adults
Multiple endocrine neoplasia type 1	*MEN1*	1–2% in adults
Familial adenomatous polyposis	*APC*	<1%
Carney complex	*PRKAR1A*	<1%
Beckwith–Wiedemann syndrome	*IGF2, H19* at the 11p15 locus	<1%
Neurofibromatosis type 1	*NF1*	<1%

2.3.3. Clinical presentation

Adrenocortical tumours present in three ways. Hormone-producing tumours are usually detected owing to hormone excess–related signs and symptoms. Some large and/or malignant tumours present with symptoms such as abdominal pain related to a tumour mass, infiltration to adjacent structures or metastatic growth. An increasing number of adrenocortical tumours are found incidentally through unrelated radiological imaging. These incidentalomas are typically hormonally inactive, although some produce subclinical quantities of corticosteroids.

Clinical syndromes caused by hormone excesses are categorised according to the hormone in question. A cortisol-producing tumour causes Cushing's syndrome, showing signs and symptoms such as central obesity, muscle weakness, diabetes, osteoporosis, hypertension and an impaired immune response.

An aldosterone-producing tumour causes primary hyperaldosteronism, also called Conn's syndrome. Excess aldosterone results in hypertension and hypokalemia with symptoms such as muscle weakness, headache and polyuria. Aldosterone is primarily produced by adenomas and only rarely by carcinomas.

The sex hormones primarily produced by adrenocortical tumours (ACTs) consist of androgens or androgen precursors. Estrogen production is very rare. Signs and symptoms of excess androgens may go unnoticed in men, whereas women suffer from hirsutism, virilisation and menstrual disorders. Sex hormone production is related to carcinomas more often than to adenomas (Lloyd et al. 2017a).

2.3.4. Pre-operative diagnostics

2.3.4.1. Biochemical measurements

If an adrenal tumour is discovered resulting from an examination associated with a hormonal disorder, adequate biochemical measurements have presumably already been performed. In the case of an incidental tumour finding, the patient should be assessed for signs and symptoms of hormonal excess. The presence of any of these signs or symptoms calls for the further biochemical assessment of the hormone in question (see Table 3). Even in cases of an asymptomatic and apparently nonfunctioning tumour, a 1-mg overnight dexamethasone (DX) test is recommended to rule out low-level 'subclinical' cortisol excess. Furthermore, some tumours produce more than just one hormone. The need to exclude phaeochromocytoma in asymptomatic cases remains unclear, although if imaging features are indeterminate and if the tumour is not clearly of an adrenocortical origin, catecholamines should be tested. Moreover, sex hormones should be measured if a carcinoma is suspected, since sex hormone production represents a frequent characteristic of an ACC (Fassnacht et al. 2016). Reference ranges and cutoff values for these measurements vary according to the provider.

Table 3. Hormones secreted by adrenal tumours, indications for measurement, suggested methods of measurement and diagnostic cutoff values. The dexamethasone (DX) suppression test values are taken from the 'Clinical Practice Guideline for the Management of Adrenal Incidentalomas' by the European Society of Endocrinology (Fassnacht et al. 2016). Other cutoff values represent those used by Helsinki University Central Hospital (HUCH).

Hormone	Indication	Method	Cutoff values
Cortisol	All patients	1-mg dexamethasone (DX) suppression test	>138 nmol/l – autonomous cortisol secretion 51–138 nmol/l – possible autonomous cortisol secretion
	If 1-mg DX test yields an abnormal result	U-free cortisol 24 h	>145 nmol/l (wide variation in cutoff values)
		P-ACTH	<10 ng/l
Aldosterone	Hypertension or hypokalemia	Aldosterone/renin ratio 24 h U-aldosterone	>800 pmol/l per µg/(l·h) >40 nmol
Catecholamines	All patients or patients without clear evidence of an adenoma	24 h U-metanephrines fractioned S-free metanephrine	U-metanephrine >1.7 µmol U-normetanephrine >4.0 µmol S-metanephrine >0.5 nmol/l S-normetanephrine >0.9 nmol/l
Sex steroids and precursors	Tumour suspicious for malignancy	S-DHEAS S-androstenedione S-hydroxyprogesterone Women: S-testosterone Men: S-estradiol	According to age and sex

2.3.4.2. Radiological imaging

The treatment of hormonally active adrenal tumours consists of resection, whilst specific diagnosis relies on histopathological examination of the surgical specimen. Hormonally inactive tumours, therefore, must be evaluated using radiological imaging. Figure 4 shows a flowchart of the evaluation process.

Unenhanced CT is the primary imaging modality used to assess the nature of adrenal tumours. In unenhanced CT, a regular shape, homogenous consistency and low density suggest a benign nature. In addition, rounded, peripheral or septal calcifications can be seen in benign lesions. An unenhanced CT attenuation value ≤10 Hounsfield units (HUs) is considered a safe threshold to diagnose a lipid-rich ACA. Tumours with benign radiological characteristics and an attenuation value ≤10 HUs require no further medical procedure. In an unenhanced CT, if a tumour presents with an attenuation value of >10 HUs or any suspicious radiological characteristics such as an irregular shape, heterogenous consistency such as haemorrhagic or necrotic areas or punctate, irregular or dystrophic calcifications, further imaging examinations are needed (Fassnacht et al. 2016).

Contrast-enhanced CT with washout measurements is performed to examine any indeterminate tumours. The basis for this imaging modality is that benign tumours enhance less and washout the contrast medium more rapidly than malignant tumours. A washout CT protocol consists of an unenhanced scan, a contrast-enhanced scan with a delay of 60 to 90 s and a delayed scan at 15 min. Absolute enhancement washout ≥60% is considered the threshold for a benign adenoma. Follow-up imaging 6 to 12 months later is recommended for such adenomas, if their diameter exceeds 4 cm. If the contrast-enhanced CT is contra-indicated, a chemical shift magnetic resonance imaging (MRI) can be performed whereby signal drop in an out-of-phase versus an in-phase image indicates a benign adenoma (Fassnacht et al. 2016).

In addition to the general decision-making guidelines, individual characteristics and the patient history should always be taken into account. If the patient has a history of malignant disease, metastasis should be considered. If on the basis of symptoms, biochemical measurements or imaging characteristics such as a high unenhanced CT attenuation value and a delayed washout a phaeochromocytoma is suspected, other imaging modalities including PET-CT may be useful (Fassnacht et al. 2016; Mayo-Smith et al. 2017 JACR).

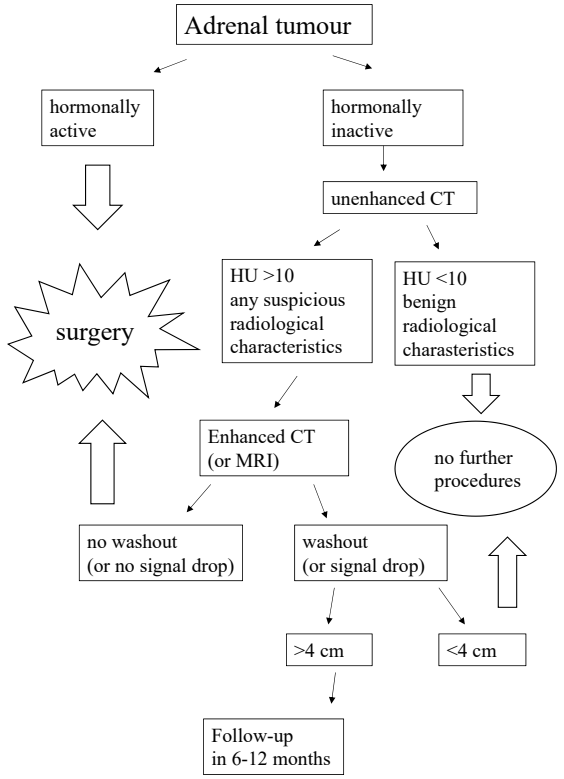

Figure 4. Flowchart of the evaluation of adrenal tumours. Modified from the 'Clinical Practice Guideline for Management of Adrenal Incidentalomas' by the European Society of Endocrinology (Fassnacht et al. 2016).

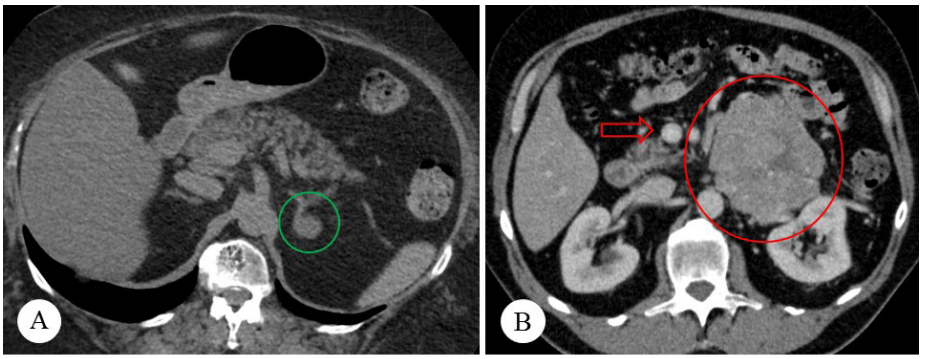

Figure 5. CT scans of adrenocortical tumours. A) Unenhanced CT of an adenoma (circled with green). The tumour is small, has a smooth contour and a homogenous consistency. B) Contrast-enhanced CT of a carcinoma (circled with red). The tumour is large, has an irregular contour and a heterogenous consistency. Enlarged lymph nodes can also be seen (red arrow). Images courtesy of abdominal radiologist Helka Parviainen, Helsinki University Central Hospital.

2.3.5. Surgical treatment

Currently, only hormonally active tumours and tumours with malignant features are surgically excised. The entire adrenal gland is typically removed. Apparently, benign tumours with a diameter <4 cm can be safely removed by laparoscopy. In case of a suspected carcinoma, an open adrenalectomy is recommended and the periadrenal fat is then removed with the adrenal gland. Complete resection is a priority and the tumour should remain intact during the operation. If the tumour invades adjacent structures, these structures should be removed en bloc with the adrenal gland. The periadrenal lymph nodes, nodes in the renal hilus and any suspicious lymph nodes should also be removed (Gaujoux et al. 2017).

2.3.6. Histopathological diagnosis

Diagnosing an adrenocortical **adenoma** (ACA) from a surgical adrenalectomy specimen is quite straightforward in most cases. Figure 6 shows a macroscopic image and microscopic images of an adenoma. Typically, an adenoma is a macroscopically yellow or orange sharply delineated tumour, the size of which rarely exceeds 5 cm. Sometimes an adenoma may contain large amounts of lipofuscin pigment, appearing black. Histologically, adenomas consist of fairly large clear cells or smaller eosinophilic cells, arranged in cords and nests with small intervening sinusoids. The clear cytoplasm results from abundant lipids dissolved during tissue processing. The lipids constitute precursors of corticosteroids. Aldosterone-producing adenomas typically present with very large clear cells and the surrounding adrenal cortex is often micronodular. Cortisol-producing adenomas usually contain smaller cells with a more eosinophilic cytoplasm, and the surrounding adrenal cortex is atrophic due to excess cortisol (Lloyd et al. 2017a).

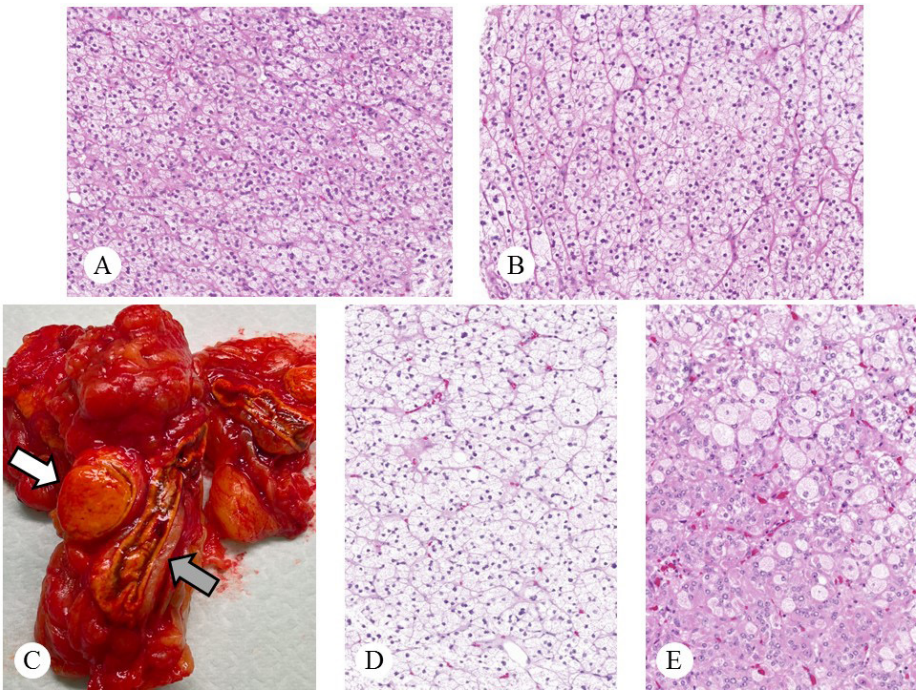

Figure 6. Adrenocortical adenoma: macroscopic and microscopic images. C) An adrenalectomy specimen with a sharply circumscribed, homogenous, yellowish adenoma (white arrow). The adrenal gland is indicated with a grey arrow. A, B, D and E) Tumour cells are arranged in cords and nests with small intervening sinusoids. A, B and D) The cells primarily have a clear cytoplasm and the nuclei are fairly small. E) A proportion of the tumour cells are eosinophilic and the nuclei are larger.

Sometimes the differential diagnosis between an ACA and nodular hyperplasia may present a problem. Adrenocortical hyperplasia typically affects both adrenal glands and, thus, a single unilateral tumour upon radiological imaging favours the diagnosis of an adenoma rather than nodular hyperplasia. However, a hyperplastic dominant nodule can sometimes grow to become comparatively large and mimic an adenoma. In addition, aldosterone-producing adenomas frequently present with accompanying micronodular hyperplasia, adding to the confusion. Thus far, no definitive histological or IHC markers exist to differentiate between hyperplasia and adenoma. In some cases, only post-surgical hormone measurements verify the diagnosis (Dekkers et al. 2014; Hellman et al. 2019).

A typical adrenocortical **carcinoma** (ACC) is a large, brown or reddish tumour with areas of haemorrhage and necrosis. A carcinoma might invade adjacent tissues and organs. ACC is characterised by malignant histological features including nuclear atypia, eosinophilic cytoplasm, a diffuse growth pattern, high mitotic activity, atypical mitoses and invasion of vascular structures and the tumour capsule. Figure 7 provides a macroscopic image and microscopic images of a carcinoma. Nonetheless, not all carcinomas present with abundant malignant features and tumours with

intermediate characteristics cause diagnostic problems (Lloyd et al. 2017a). To address the need to differentiate between benign and malignant adrenocortical tumours, several diagnostic criteria have been proposed and are addressed further in the section below on histopathological markers. The mitotic count can be used to grade carcinomas into two prognostically relevant groups: low grade <20 mitoses per 50 high-power fields (HPF) and high grade ≥20 mitoses per 50 HPF (Giordano et al. 2011; Weiss et al. 1989).

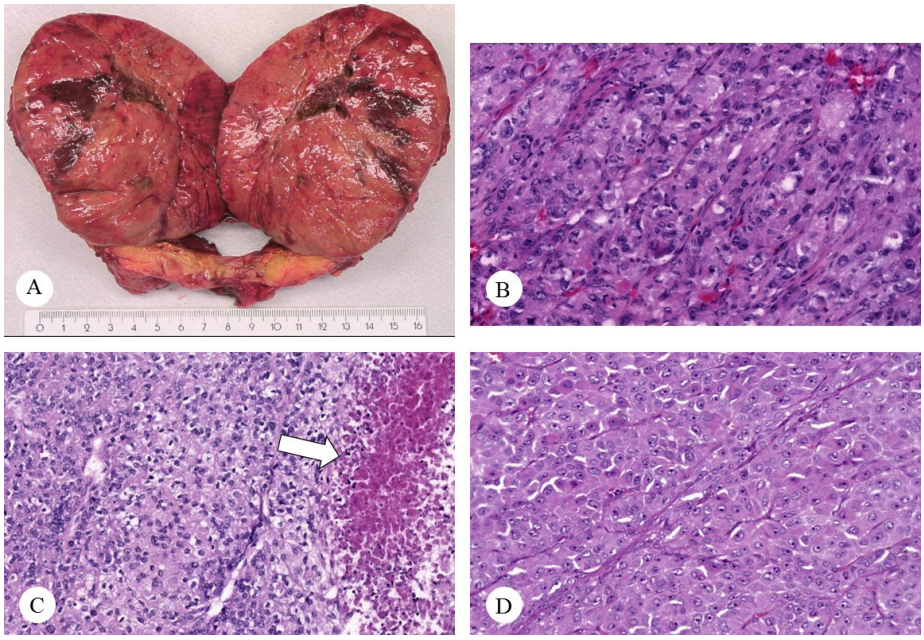

Figure 7. Adrenocortical carcinoma: macroscopic and microscopic images. A) An adrenalectomy specimen, where a large tumour is cut in half. The tumour has a heterogenous consistency, variable colour and brown haemorrhagic areas. B, C and D) Tumour cells are fairly large, and primarily have an eosinophilic cytoplasm and pleomorphic nuclei. C) A necrotic area can be seen, indicated by the white arrow.

Histological variants of ACTs include oncocytic, myxoid and sarcomatoid variants, all of which are uncommon. **Oncocytic tumours** are characterised by large cells with a fine granular eosinophilic cytoplasm composed of abundant mitochondria. The nuclei of oncocytic tumours are large and have prominent nucleoli. Oncocytic tumours present with a diffuse growth pattern. A tumour can be defined as oncocytic if at least 50% of the cells are oncocytes. An oncocytic tumour can be a mixed tumour (50–90% oncocytes) or completely oncocytic (>90%). Most oncocytic tumours present with a favourable prognosis (Lloyd et al. 2017a).

Myxoid tumours are rare. The classification and definition of myxoid ACTs remain debated, since small amounts of degenerative myxoid change can be seen in many tumour types. In short, myxoid change is primarily associated with ACC,

and very rarely with adenomas. The significance of myxoid change for the prognosis of the tumour patient remains unclear, since large enough series have not been studied (de Krijger et al. 2012).

A sarcomatoid ACC is a variant that can present in a monophasic or biphasic form. Similar to many other carcinomas, a sarcomatoid component represents a loss of differentiation and is, thus, associated with a worse prognosis. A monophasic sarcomatoid ACC can be difficult to differentiate from a sarcoma (Lloyd et al. 2017a).

In some instances, the origin of an adrenal tumour remains unclear and must be verified to exclude an adrenomedullary tumour or metastasis to the adrenal gland. This distinction is accomplished through **immunohistochemistry** (IHC). The only protein expressed exclusively in adrenocortical tissue is steroidogenic factor 1 (SF1). Table 4 summarises the other proteins frequently used to differentiate between adrenocortical and other neoplasia.

Table 4. Immunohistochemistry panel for the differential diagnosis of primary adrenal tumours and adrenal metastases, including examples of useful markers.

Neoplasia	Steroidogenic factor 1	Pan-cytokeratin	MART1/ MelanA	Chromo-granin A	Inhibin α	CEA
Primary adrenocortical neoplasia	+	+/–	+	–	+	–
Phaeochromocytoma or paraganglioma	–	–	–	+	–	–
Renal cell carcinoma	–	+	–	–	–	–
Adenocarcinoma	–	+	–	–	–	+/–
Melanoma	–	–	+	–	–	–
Neuroendocrine tumour	–	+	–	+	–	+/–
Urothelial carcinoma	–	+	–	–	–	+/–
Hepatocellular carcinoma	–	+	–	–	–	–

When diagnosing an ACC, its stage must be determined. The European Network for the Study of Adrenal Tumours (ENSAT) staging system, summarised in Table 5, is recommended by WHO. In addition to histopathological examination, radiological imaging is necessary to determine the stage. The minimum requirement is CT or MRI scanning of the thorax, abdomen and pelvis.

Table 5. The ENSAT staging system according to the TNM status (Fassnacht et al. 2009)

ENSAT stage	T	N	M
I	T1	N0	M0
II	T2	N0	M0
III	T1–T2	N1	M0
	T3–T4	N0–N1	M0
IV	T1–T4	N0–N1	M1

T1, tumour ≤ 5 cm; T2, tumour > 5 cm; T3, infiltration into the surrounding tissue; T4, tumour invasion into the adjacent organs or venous tumour thrombus in the vena cava or renal vein; N0, no positive lymph nodes; N1, positive lymph node; M0, no distant metastases; M1, presence of distant metastases.

2.3.7. Post-operative management of adrenocortical carcinoma (ACC)

When planning treatment for an ACC patient, the tumour stage, resection status, proliferation using the Ki-67 index and the patient's general condition should be taken into account. Complete resection of the tumour is required to cure the patient. In the case of an incomplete resection, local treatment methods, such as radiation, radiofrequency ablation, cryoablation, microwave ablation or chemoembolisation can be used. Even when the tumour is completely removed, adjuvant therapy is used in most cases to prevent recurrence (Fassnacht et al. 2018).

2.3.7.1. Adjuvant therapy by mitotane

Mitotane is an adrenolytic agent, a derivative of the insecticide DDT, and has been used to treat ACC since 1959. The action of mitotane partly relies on the inhibition of steroidogenic enzymes. The mechanism of its selective damaging effect on adrenocortical tissue remains unknown. First, mitotane was only used for inoperable or disseminated cases of ACC, but was later employed as an adjuvant therapy to prevent recurrence and to reduce ACC mortality. Studies on the effect of mitotane have yielded conflicting results, largely because of the retrospective nature of such studies and bias related to patient selection. No randomised prospective clinical trials have been published on mitotane as an adjuvant treatment. However, in a meta-analysis by the European Society of Endocrinology (ESE), mitotane appears to carry a favourable effect both on recurrence and mortality (Fassnacht M 2018).

The therapeutic response of mitotane is achieved at a plasma level of 14 mg/l, whereas a plasma concentration above 20 mg/l results in toxicity. Mitotane plasma levels are, thus, monitored rigorously during treatment. Even within the therapeutic window, mitotane treatment causes frequent side effects (Paragliola et al. 2018).

In the ESE 'Clinical Practice Guideline', mitotane treatment is recommended for patients with metastatic or residual disease and as an adjuvant treatment for

patients with a high risk of recurrence (stage III, proliferation >10%). For patients with a completely resected local tumour and a low risk of recurrence (stages I–II, proliferation ≤10%), mitotane adjuvant treatment should be considered on a case-by-case basis (Fassnacht et al. 2018).

2.3.7.2. Disseminated disease

The prognosis for recurrent or metastatic ACC is poor and no curative treatment is available. Therefore, treatment of disseminated ACC aims to alleviate symptoms and extend patient survival. Decisions should always be made individually, according to the expert clinical judgement from a multidisciplinary team.

The most common sites to which ACC spreads include the periadrenal tissue, lymph nodes, lungs, liver and bones (Abiven et al. 2006). Both locally recurrent ACC and singular metastases in the liver or lungs can be treated surgically. However, surgical resection is only recommended if a radical result can be achieved. Palliative or debulking surgery of disseminated disease is not beneficial (Gaujoux et al. 2017). If complete resection is impossible, other local treatment methods, such as radiation, radiofrequency ablation, cryoablation, microwave ablation or chemoembolisation can be used.

For any form of disseminated disease, mitotane treatment is recommended. Furthermore, in aggressive cases of ACC, a chemotherapy combination consisting of etoposide, doxorubicin and cisplatin (EDP) can be beneficial (Fassnacht et al. 2018).

2.3.7.3. Follow-up

After a complete ACC resection, regular follow-up should include clinical assessment, radiological imaging and biochemical evaluation for hormonal excess. The timeline for follow-up is every 3 months for the first 2 years, and every 3 to 6 months for the following 3 years. After 5 years, the follow-up frequency can be adjusted. CT or MRI scans of the chest, abdomen and pelvis should detect regional recurrence or spread in the abdominal cavity, as well as metastasis to the liver and lungs. More extensive imaging may be performed for suspected metastasis in other locations. Signs and symptoms of tumour spread as well as tolerance for possible adjuvant therapy is assessed clinically during each visit. In the case of a hormonally active primary tumour, the particular hormone should be assessed biochemically (Fassnacht et al. 2018).

2.4. Markers of malignancy

When considering the distinction between benign and malignant tumours, **malignancy** must be defined. Malignant characteristics such as invasion, metastasis and recurrence accompany ACCs. In the presence of any of these characteristics, a tumour can be defined as malignant with a degree of certainty. In the absence of these characteristics, the nature of the tumour must be assessed using malignancy associated markers, predicting malignancy with varying specificity. In addition, amongst malignant tumours, a **prognostic classification** is necessary in order to delineate the appropriate treatment strategy for each patient. Different markers have been studied to reliably diagnose ACTs and to evaluate prognosis. The most relevant markers are categorised as histopathological markers, proteins and genetic markers of malignancy, although significant overlap exists between these three categories. Before proceeding, however, I address a quantitative marker of malignancy not fitting in any of the above-mentioned categories.

A large **tumour size** associates with malignancy. Adenomas are primarily less than 5 cm in diameter. For a carcinoma, however, the median size exceeds 10 cm, and rarely measures less than 4 cm. Thus, size can be used to predict malignancy. In the preoperative evaluation of adrenal tumours, a cutoff value of 4 cm is commonly used to predict malignancy and favour surgical treatment. Furthermore, in the TNM staging, a tumour size of 5 cm distinguishes between T1 and T2. However, rare large adenomas and small carcinomas impair the diagnostic power of the tumour size as an independent parameter in predicting malignancy (Fassnacht et al. 2016).

2.4.1. Histopathological markers

The same histopathological features associated with malignancy in any tumour type, including cytological atypia, growth pattern, mitotic activity, necrosis and invasion, also serve as markers of malignancy in ACTs. Diagnostic scoring systems and algorithms combining these features have been developed to diagnose ACTs accurately as well as to predict the prognosis of tumour patients.

The Weiss scoring system is the most widely used histological diagnostic system in use globally, and serves as the reference system for other diagnostic methods and scorings. The system was introduced by Dr. Weiss in 1984, a system he further refined in 1989. The Weiss system includes nine histological criteria, all associated with malignant behaviour and a worse prognosis. Table 6 summarises the criteria of the Weiss system. A tumour receives one point for each criterion it fulfills. The presence of three or more criteria indicates a carcinoma. In addition, the total Weiss score inversely correlates with survival, whereby the higher the score, the worse the prognosis (Weiss 1984; Weiss et al. 1989).

Table 6. The Weiss (1984) histological criteria.

Nuclear atypia*
Mitoses >5/50 high-power field
Atypical mitoses
Eosinophilic cytoplasm ≥75% of cells
Diffuse growth >30%
Necrosis
Sinusoidal invasion
Venous invasion
Capsular invasion

* According to the Fuhrman grade: grades 3–4 define nuclear atypia (Fuhrman et al. 1982).

The Weiss scoring system is quite sensitive in diagnosing a carcinoma. Furthermore, the Weiss score can be determined from basic hematoxylin and eosin (HE) stained slides, whereby no special stains or IHC is necessary. However, the Weiss system carries several weaknesses. One lies in the specificity, since some tumours with a low score on the malignant scale never recur or metastasise. In addition, the Weiss scoring system suffers from subjectivity. Some criteria, such as diffuse growth and sinusoidal invasion, are particularly difficult to assess, causing interobserver variation in their interpretation, thus limiting the reliability of diagnosis (Aubert et al. 2002; Tissier et al. 2012).

To render the diagnostic process more simple and objective, Aubert et al. (2002) introduced a modification to the Weiss system. The most objective criteria of the Weiss system were included in this new system, consisting of a total of five criteria. These criteria were then weighted using a regression parameter to achieve the best correlation with the Weiss score. The calculation, 2x mitotic rate + 2x eosinophilic cytoplasm + atypical mitoses + necrosis + capsular invasion, yields the revised Weiss score, summing values from 0 to 7. Scores from 3 to 7 indicate a malignancy. This revised Weiss system correlated significantly with the Weiss system ($r = 0.98$). Thus, despite representing a more objective system, the revised Weiss system only achieved the same diagnostic power as the original Weiss system. The specificity of the Weiss system remained unimproved.

Volante et al. (2009) introduced a diagnostic algorithm for ACTs, based on examining the reticulin structure of the tumour. In a carcinoma, the reticulin framework is disrupted and this feature serves as the first step in the algorithm. In addition, to qualify as a carcinoma, the tumour must fulfill at least one of the following criteria: mitosis frequency >5/50 HPF, the presence of necrosis or vascular invasion. The reticulin algorithm was compared to the Weiss score and appeared

equally sensitive and specific. Duregon et al. (2013) verified the interobserver reproducibility of the reticulin stain evaluation in an extensive study.

Other diagnostic systems include scoring systems developed by Hough et al. (1979) and van Slooten et al. (1985). The Hough system combines histological with clinical criteria. The van Slooten system, in comparison, relies on purely histological criteria with weighted values. Neither of these systems has been widely used in clinical practice.

The diagnostic and prognostic assessment of oncocytic ACTs is more challenging than that of conventional tumours. The Weiss scoring system overestimates malignancy, since three of the Weiss criteria (eosinophilic cytoplasm, nuclear atypia and diffuse growth) are fulfilled by definition in oncocytic tumours. When using the Weiss score to define a malignancy, oncocytic tumours carry a more favorable outcome and prognosis than conventional carcinomas. To improve the diagnostic accuracy for oncocytic tumours, Bisceglia et al. (2004) proposed a modified diagnostic system. The Lin–Weiss–Bisceglia system combines six Weiss criteria with the size of the tumour. This system includes three major criteria: a mitotic rate >5/50 HPF, atypical mitoses and venous invasion. In addition, the system has four minor criteria: necrosis, sinusoidal invasion, capsular invasion and a tumour size >10 cm and/or weight >200 g. The presence of one major criterion indicates malignancy, whereas the presence of one minor criterion indicates an uncertain malignant potential. The absence of all criteria indicates a benign tumour. WHO recommended this system in 2017.

Myxoid ACTs remain rare, and only single cases or small series have been reported. Myxoid change appears in varying degrees in these tumours (de Krijger et al. 2012). Papotti et al. (2010) suggested categorising myxoid tumours into two groups, which feature different histological characteristics and the distribution of myxoid change. Specifically, these consist of predominantly myxoid tumours with a nested, glandular or trabecular growth pattern and mild cytological atypia (type 1), and conventional ACCs with small areas of myxoid change, diffuse growth and the same histological features as in the conventional component (type 2). Papotti et al. (2010) further suggested divergent differentation paths for these tumour types based on the presence of neurofilament proteins in type 1 tumours. The diagnostic problem lies in the fact that type 1 tumours present with a much worse prognosis than expected based on the mild cytological atypia and lack of a diffuse growth pattern. It appears that these type 1 tumours indeed form a particular tumour group and that these tumours can progress to a more malignant form. The Weiss scoring system is, thus, not appropriate for myxoid tumours.

Mitotic activity emerges as a significant marker of malignancy in all diagnostic systems. A high mitotic count associates with both malignancy and a worse prognosis (Assie et al. 2007; Blanes et al. 2007; Miller et al. 2010; Stojadinovic et al. 2002; Volante et al. 2009; Weiss et al. 1989). However, no cutoff level for the mitotic

activity can define a malignancy with a sufficient degree of accuracy. In the Weiss scoring system, a cutoff value 5/50 HPF was set for the mitotic activity criterion, with the same value used in the revised Weiss score (Aubert et al. 2002), in the reticulin algorithm (Volante et al. 2009) and in the Lin–Weiss–Bisceglia system for oncocytic tumours (Bisceglia et al. 2004). The mitotic count has been used to define low- and high-grade carcinomas, with a cutoff of 20/50 HPF (Weiss et al. 1989; Giordano et al. 2011). Adding the grade to the ENSAT staging system enhanced its accuracy to predict recurrence and survival amongst carcinoma patients (Miller et al. 2010). This Ann Arbor modification of the ENSAT staging system has not been applied in clinical practice thus far, although the ESE practical guidelines for managing ACC recommend determining the exact mitotic count for every ACC (Fassnacht et al. 2018).

ACTs amongst children remain rare, and most often associate with hereditary tumour syndromes. The diagnostic and prognostic systems designed for adult patients do not adapt well to children, since they overestimate malignancies in children. In other words, with a histologically similar tumour, children have a better prognosis than adults (Michalkiewicz et al. 2004; Wieneke et al. 2003). A diagnostic system based on the tumour weight, invasion to adjacent tissues and the presence of metastasis has been proposed for children's tumours (Dehner et al. 2009).

2.4.2. Immunohistochemical (IHC) markers

The proliferation activity is a strong predictor of malignancy as well as the clinical outcome in ACT patients (Beuschlein et al. 2015; Morimoto et al. 2008; Soon et al. 2009; Terzolo et al. 2001; Wachenfeld et al. 2001). Proliferation is assessed by immunohistochemical (IHC) staining of the cell cycle protein **Ki-67**. Although carcinomas present with a higher proliferation than adenomas, setting an applicable threshold to differentiate between adenomas and carcinomas proved difficult, since some metastatic carcinomas show a surprisingly low proliferation. A cutoff value of 5% can diagnose a carcinoma with a high specificity, but with a poorer sensitivity (Arola et al. 2000; Soon et al. 2009; Wachenfeld et al. 2001).

Proliferation has proved particularly useful for prognostic purposes. In 2018, ESE concluded that in localised ACC (stages I–III) the main factors predicting recurrence consisted of the tumour stage, the resection status (R0/R1) and the proliferation. In its clinical practice guideline, ESE recommends categorising these patients into two groups. The low- to moderate-risk group includes patients with stage I or II, R0 and Ki-67 ≤10%. The high-risk group includes patients with stage III, R1 or Ki-67 >10%. Adjuvant therapy is recommended for all high-risk patients. Therefore, by associating with prognosis, proliferation influences the treatment strategy (Fassnacht et al. 2018). However, Ki-67 is not a predictive factor when it comes to mitotane or chemotherapy efficacy.

In addition to the immunostaining of Ki-67, **insulin-like growth factor 2 (IGF-2)** is the only IHC marker recommended by WHO to distinguish between adenomas and carcinomas (Lloyd et al. 2017a). Indeed, IGF-2 expression appears to associate with malignancy in a number of studies (Heaton et al. 2012; Soon et al. 2009, Wang et al. 2014). The physiological role of IGF-2 is related to growth and differentiation during gestation. The *IGF-2* gene is located at the imprinted locus 11p15. Structural and functional abnormalities at 11p15 are associated with ACC (Gicquel et al. 1997), as well as with IGF-2 immunostaining. That is to say, activation of this developmental gene following the embryological period results in oncogenesis. Interestingly, IGF-2 expression has proved particularly useful in predicting malignancy in tumours with a low proliferation (Soon et al. 2009).

An increased **ß-catenin** IHC expression appears in all ACTs, although staining is more intense in carcinomas than in adenomas (Tissier et al. 2005). The localisation of positivity also differs between adenomas and carcinomas. In adenomas, ß-catenin primarily expresses in the cytoplasm and cell membrane, whereas in carcinomas, nuclear staining is prominent (Gaujoux et al. 2011). Nuclear staining has been associated with an activating mutation to the ß-catenin gene and a worse prognosis in carcinomas (Kovach et al. 2015). Immunostaining of ß-catenin also indicates an activated Wnt/ß-catenin pathway, which I will return to below.

Aberrant **protein 53 (p53)** IHC staining identifies a group of ACC patients with a poor prognosis (Waldmann et al. 2012). Only a fraction of these tumours harbour a mutation of the tumour suppressor gene p53, although even some germline mutations are detected amongst sporadic ACCs. Aberrant p53 IHC associates with a higher proliferation in ACC.

The expression of **cyclin E** correlates with both malignancy and adverse prognosis in ACT (Tissier et al. 2004). Cyclin E is a progressor of the cell cycle and its overexpression has been described in various neoplasias. However, its particular role in ACC remains unclear. Tissier F et al. (2004) found no correlation between cyclin E expression and the overproduction of IGF-2.

Steroidogenic factor 1 (SF1) immunostaining is used in the differential diagnosis of adrenal tumours to indicate an adrenocortical origin. SF1 is expressed in both the foetal and adult adrenal cortex. Recently, staining intensity was found to associate with a worse prognosis in ACC (Duregon et al. 2013; Sbiera et al. 2010).

2.4.3. Molecular changes in adrenocortical neoplasia

The genetic background of adrenocortical neoplasias has been examined with increasing intensity in recent decades. The motivation for this research focuses on four primary goals: to understand the pathogenesis of these tumours, to more accurately diagnose these tumours, to classify tumours according to prognosis and, most recently, to identify targets for individualised therapies.

With the advances in genetic methodology, an increasing amount of information has allowed for the integrated genomic characterisation of adrenocortical neoplasias. Regardless of the method, recurrent themes stand out: the expression of IGF-2, cell cycle–related and steroidogenic genes, the number of chromosomal changes, an alteration in p53 and the activation of the Wnt/ß-catenin pathway can discern tumours according to malignancy and prognosis. Figure 6 illustrates these primary lines, associating transcriptome-based classification to the molecular features of the tumours. Interestingly, recent findings in microRNA expression and the DNA methylation of adrenocortical neoplasias also agree with this classification. Furthermore, this molecular classification validates the diagnostic and prognostic IHC markers previously described (Assie et al. 2014). The molecular mechanisms of adrenocortical tumourigenesis relevant to this research are discussed further below.

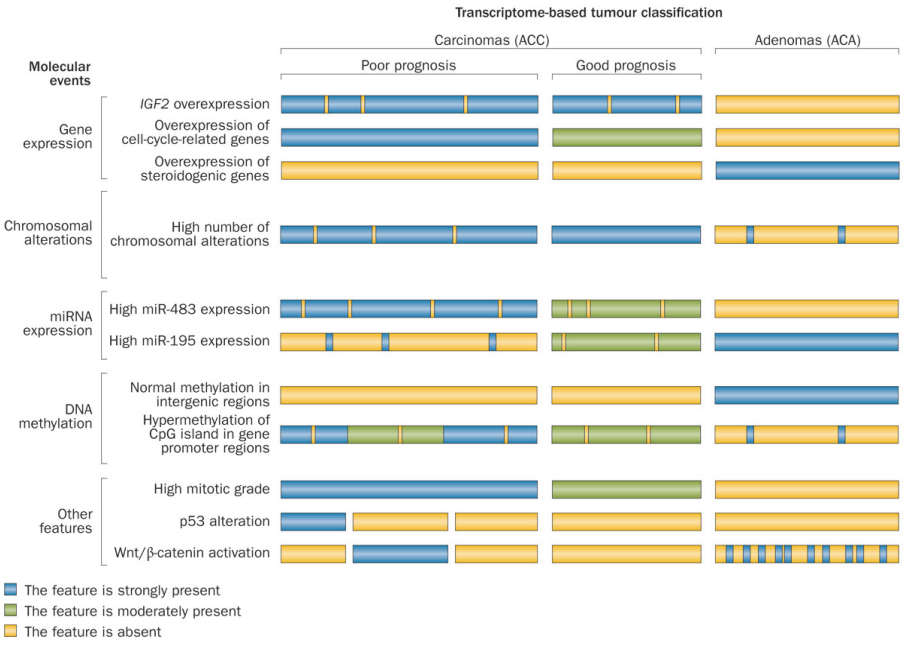

Figure 8. Molecular classification of adrenocortical tumours according to genomic information (Assie et al. 2014). Published with permission from Macmillan Publishers Ltd.

2.4.3.1. Wnt/ß-catenin pathway

The role of the Wnt/ß-catenin pathway in adrenocortical tumourigenesis is noteworthy since it is involved in both benign and malignant tumours. In addition, it is mutually exclusive with p53 alterations. The Wnt/ß-catenin pathway transmits signals from outside the cell through a chain of protein interactions to the nucleus, leading to the regulation of gene transcription related to cell proliferation, differentiation and apoptosis. In short, the binding of Wnt to a cell-membrane receptor leads to the accumulation of ß-catenin in the cytoplasm and its translocation to the nucleus, where it acts as a transcriptional coactivator. Without Wnt, ß-catenin degrades. Under physiological conditions, Wnt signaling is closely regulated. The activation of the pathway can, in principal, result from various alterations concerning the system, such as activating mutations or the upregulation of its components, inactivating mutations or downregulation of its inhibitors or alterations in the pathways interacting with the Wnt/ß-catenin pathway (Clevers et al. 2012). In ACTs, two mechanisms activating the Wnt/ß-catenin pathway have been identified. First, activating mutations of the ß-catenin gene *CTNNB1* have been associated with tumourigenesis in both benign and malignant tumours. These are all mutations of exon 3, which protect ß-catenin from targeted degradation (Gaujoux et al. 2011; Tissier et al. 2005). The second established mechanism was discovered via studies of patients with familial adenomatous polyposis. These patients have an inherited heterozygous germline mutation of the *APC* gene. In addition to developing numerous intestinal polyps, they also show an increased incidence of desmoid tumours (De Marchis et al. 2017) and ACTs (Marchesa et al. 1997). This results from the APC protein being part of the 'destruction complex' of ß-catenin and, thus, inactivated APC fails to degrade ß-catenin. However, inactivating mutations in *APC* are primarily associated with ACAs and rarely with carcinomas, whilst *APC* mutations involve sporadic ACTs rather infrequently (Gaujoux et al. 2010). Increased IHC staining of ß-catenin appears in basically all ACTs, with cytoplasmic staining predominating in adenomas and nuclear staining in carcinomas. Yet, mutations of the ß-catenin gene and *APC* only account for a small proportion of these cases (Bonnet et al. 2011; Gaujoux et al. 2011). Therefore, other unidentified mechanisms must be involved in the Wnt activation in ACTs.

2.4.3.2. C-myc: A multifunctional transcription factor and a proto-oncogene

C-myc is a transcription factor expressed universally in all cells, which induces the expression of various target genes involved in proliferation, cell growth, loss of differentiation and apoptosis. Under physiological conditions, c-myc gene expression is precisely regulated. C-myc is activated by mitogens, growth factors and via multiple signaling pathways. Both the overexpression of c-myc and the deregulation

of its expression or function can lead to a cell's malignant transformation. The overexpression of c-myc was first detected in Burkitts lymphoma (Dalla–Favera et al. 1982) and has since been described in many cancers (Bai et al. 1994; Calcagno et al. 2009; Pietilainen et al. 1995).

Unsurprisingly for a transcription factor, the c-myc protein is normally evident in the nuclei of tumours presenting with an over- or deregulated expression of the c-myc gene. Nonetheless, cytoplasmic c-myc is also evident in some neoplasias (Bai et al. 1994; Calcagno et al. 2009; Pietilainen et al. 1995; Ruzinova et al. 2010), correlating with cancer aggressiveness. The role of cytoplasmic c-myc has been studied rigorously by Conacci-Sorrell et al. (2010; 2014), who presented a truncated, transcriptionally inactive cytoplasmic c-myc protein called myc-nick, which promotes cancer cell survival and augments cancer cell motility. They also showed that myc-nick expresses in a wide range of tumours. Myc-nick is generated in response to metabolic and cytotoxic stress by calpain-mediated proteolysis, under which conditions the full-length c-myc would cause cell death by apoptosis.

Surprisingly, in ACCs, the underexpression of c-myc has been described as a pathogenic event (Szabo et al. 2010; 2011). A decreased c-myc expression has been suspected in the pathway analysis of gene expression microarray and comparative genome hybridisation studies, indicating that it is connected to deletions in chromosome 8q24. However, no significant losses or gains in chromosome 8q24 harbouring the c-myc gene nor any amplification nor rearrangement of the gene have been found in adrenocortical neoplasias (Giordano et al. 2009; de Reynies et al. 2009). Interestingly, the Wnt/beta-catenin pathway, one of the signaling pathways leading to the induction of c-myc expression, has been connected to the development of adrenocortical neoplasias, offering a possible connection between c-myc and adrenocortical neoplasia (Berthon et al. 2010; Bonnet et al. 2011; Durand et al. 2011; Gaujoux et al. 2011; Parviainen et al. 2013; Ragazzon et al. 2010; Tissier et al. 2005).

In adrenocortical neoplasia, Liu et al. (1996; 1997) studied c-myc gene expression. They found that c-myc mRNA expresses abundantly in normal adrenocortical tissue, hormonally inactive carcinomas and cortisol- or aldosterone-secreting adenomas. Hormonally active carcinomas and testosterone-producing adenomas presented with a decreased level of c-myc mRNA. Furthermore, they also found a solid correlation between the mRNA expression and nuclear IHC staining. Suzuki et al. (1992) studied the IHC expression of the c-myc protein in ACTs. They observed the expression of the c-myc protein in the nuclei of all 15 tumours studied. Furthermore, in carcinomas, c-myc staining also occurred in the cytoplasm.

2.4.3.3. Isocitrate dehydrogenase (IDH): A metabolic enzyme and a proto-driver-oncogene

The oncogenic capacity of mutated isocitrate dehydrogenase (IDH) was first identified in gliomas (Parsons et al. 2008). This finding was unprecedented, since IDH is a metabolic enzyme. In its wild type, IDH is responsible for the oxidation of isocitrate to α-ketoglutarate and the conversion of $NAD(P)^+$ to NAD(P)H. The mutated form of IDH prompts neomorphic activity, resulting in the conversion of α-ketoglutarate to an oncometabolite, D-2-hydroxyglutarate. The subsequent accumulation of the oncometabolite results in epigenetic dysregulation through inhibition of α-ketoglutarate-dependent histone and DNA demethylases, and blocking cellular differentiation (Chowdhury et al. 2011; Koivunen et al. 2012). IDH exists in three isoforms: 1, 2 and 3. Oncogenic mutations have been detected in isoforms IDH1 and 2, and following their recognition in gliomas, mutations have been reported in several neoplasias (Amary et al. 2011; Borger et al. 2012; Mardis et al. 2009; Murugan et al. 2010). All reported pathogenic mutations of IDH1 and 2 are heterozygous missense point mutations that alter the conserved binding site of the homodimer enzyme. These most often occur at codon R132 in IDH1 and codons R140 or R172 in IDH2 by replacing arginine with another amino acid.

In gliomas, IDH mutation status represents an important diagnostic and prognostic feature identified through routine clinical practice. IDH mutations occur in most lower-grade gliomas and secondary glioblastomas, indicative of a distinctive pathogenesis and a better prognosis compared to primary glioblastomas. A specific R132H mutation to IDH1 accompanies approximately 80% of all IDH mutations in gliomas (Hartmann et al. 2009). To improve the diagnostic procedure for gliomas, Capper et al. (2009) developed an antibody that binds to the mutated site of IDH1 R132H. This mutation-specific antibody can be used to detect the mutated protein from formalin-fixed paraffin-embedded tissues using IHC. Thus far, IDH mutations have not been reported in ACTs.

3 AIMS OF THE STUDY

The primary aim of this study was to identify diagnostic preoperative and postoperative markers of malignancy in ACTs to achieve tailored management of patients with adrenal lesions.

The detailed aims were as follows:

1. To identify an optimal unenhanced CT attenuation value to depict tumours requiring further examination. To achieve this aim, the correlation between unenhanced CT attenuation value and the specific histopathology, as well as the proportion of lipid-poor eosinophilic cells in ACTs was examined.

2. To optimise a scoring system for ACTs to predict their metastatic potential. To achieve this aim, the diagnostic power of the Weiss scoring system in relation to our cohort needed to be determined.

3. To study the role of c-myc and other cell cycle–related proteins in ACTs, aiming to elucidate their role in adrenocortical carcinogenesis and, thus, testing their potential use as biomarkers for malignancy.

4. To study the prognostic and predictive role of IDH1 R132H IHC staining in ACTs, as well as to identify the genetic alterations behind the positive staining result.

4 MATERIALS AND METHODS

4.1. Patient cohorts and clinical data

Figure 9 and Table 7 summarise the patient and tumour cohorts. Across all studies, our cohorts included adult patients who underwent surgery for a primary ACT in the Department of Surgery at Helsinki University Central Hospital (HUCH). Tumour specimens were stored in the archives of the Department of Pathology at the University of Helsinki, and were identified from a pathology database. Clinical data were collected from patient records. The functional status of the tumours was based on the presence of a clinical and/or biochemical adrenocortical hypersecretion. Survival and cause of death data were collected from the Population Register Centre and Statistics Finland.

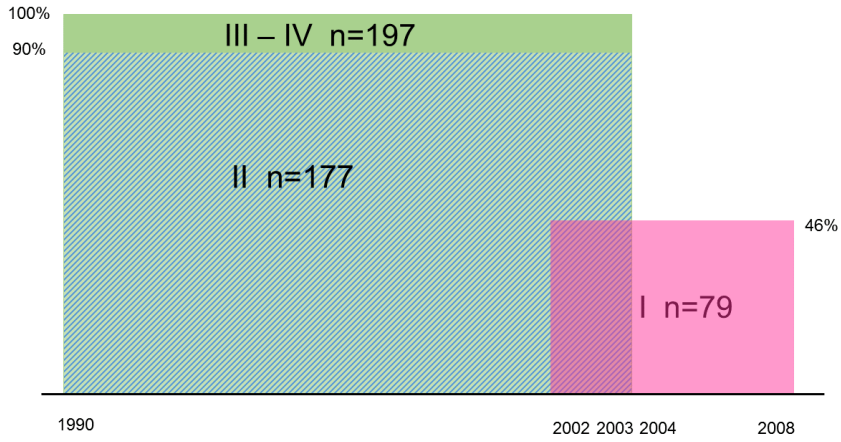

Figure 9. Tumour cohorts in studies I–IV. Study I: 79 tumours operated on between 2002 and 2008. Study II: 177 tumours operated on between 1990 and 2003. Studies III and IV: 197 tumours operated on between 1990 and 2003.

Table 7. Clinical and histological characteristics of the study cohorts in the original publications.

Study		I	II	III–IV
Patients		78*	175**	195**
Tumours	all	79	177	197
	local	76	163	183
	metastatic	3	14	14
Age (years)	range	18–83	24–82	24–82
	mean	56	53	54
	median	57	54	55
Gender	male	24	58**	66**
	female	54*	117**	129**
Side	right	35	93	101
	left	44	84	96
Size (cm)	range	0.8–19.3	0.5–28.0	0.5–28.0
	mean	3.4	3.4	3.4
	median	2.6	2.0	2.0
Weiss score	0–2	68	147	166
	3–9	11	30	31
Hormonal secretion				
Aldosterone	n	34	92	96
	%	43	52	49
Cortisol	n	26	68	74
	%	33	38.4	38
Androgens	n	2	11	11
	%	3	6	6
Inactive	n	18	20	29
	%	23	11	15

*One female had two separate local tumours.
**One male and one female had two separate local tumours.

In study I, patients operated on between 2002 and 2008 were identified from the pathology database. The study cohort included tumour patients who had appropriate preoperative unenhanced CT scans available for retrospective re-evaluation. Amongst 171 excised tumours, 79 tumours (78 patients, including one patient with two separate benign tumours) were included in this study. In this study, tumour size was based on information in the Department of Radiology database. To compare lipid-rich adenomas, lipid-poor eosinophilic adenomas and malignant tumours,

the tumours were divided into three groups according to the lipid content and the level of malignancy. Group 1 included lipid-rich adenomas, group 2 consisted of lipid-poor eosinophilic adenomas and group 3 consisted of histologically malignant tumours. Here, one of the Weiss criteria (<25% lipid-rich cells) was used to define a lipid-poor eosinophilic adenoma. Two pathologists evaluated the percentage of lipid-poor eosinophilic cells in each tumour.

In studies II through IV (IHC), patients who underwent surgery between 1990 and 2003 were identified. This series comprised 195 patients with 197 tumours, whereby two patients had two separate tumours. Studies III and IV included all 197 tumours.

In study II, 20 patients who died of causes unrelated to the ACT within five years of resection, were excluded. This ensured a minimum follow-up time of five years for each patient. The final study cohort, thus, included 175 patients with 177 tumours.

Study IV (genetics) used tumour material in the genetic analyses, as illustrated by Figure 10. For amplicon-based hotspot panel sequencing of the *IDH1* gene, we selected 10 tumours with a Weiss score of 3 to 9: 5 tumours which exhibited a strong positive and 5 featuring a negative IDH1 R132H IHC staining. For *IDH1* exon sequencing from the FFPE tumour material, we chose an additional 10 tumours: 5 tumours with a Weiss score of 0 to 2 with a positive IDH1 R132H IHC stain and 5 tumours with a Weiss score of 3 to 9 with a low positivity. Thus, 20 tumours in total were selected, for 16 of which fresh-frozen tumour material was available for *IDH1* exon sequencing.

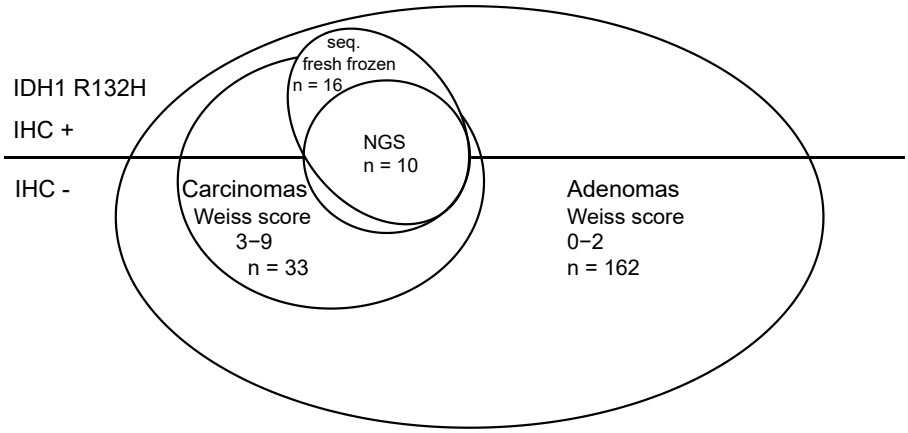

Figure 10. Tumour material in study IV, according to the histological malignancy and IDH1 R132H immunohistochemical (IHC) staining. Amongst the 195 tumours for which IDH1 R132H IHC was available, 162 tumours were adenomas (Weiss score 0–2) and 33 tumours were carcinomas (Weiss score 3–9). The horizontal line indicates the IDH1 R132H IHC staining of the tumours: positive vs. negative. Smaller circles show the cases selected for amplicon-based hotspot panel sequencing (NGS) and the fresh-frozen samples used for the exon sequencing.

4.2. Radiological imaging

CT scans were performed using 13 different scanners at various hospitals from within the HUCH district and all data were obtained from the shared radiology database all these hospitals use. The slice thickness ranged from 2.5 mm to 7 mm. In 31 cases, thin slices (3 mm or less) were available; in 48 cases, the thickness was over 3 mm. An experienced adrenal radiologist re-evaluated the CT scans. The maximal tumour diameter was determined using a distance cursor in the axial plane. The attenuation (HU) values were recalculated using a circular region-of-interest cursor placed over the area of the tumour, avoiding the edges as well as the necrotic and cystic areas to prevent partial volume artefacts. One to three measurements were obtained for each tumour. The mean HU value was recorded.

4.3. Histopathological re-evaluation

Two pathologists reviewed HE-stained sections of archived formalin-fixed paraffin-embedded tissue samples. The histopathological diagnosis according to the Weiss criteria (Weiss 1984; Weiss et al. 1989) was determined for each tumour, which received a numeric score ranging from 0 to 9. Table 6 summarises the Weiss criteria.

4.4. Tissue microarray (TMA) blocks

Tissue microarray (TMA) blocks were constructed from archived surgical formalin-fixed paraffin-embedded specimens. Representative areas of each tumour were chosen from HE-stained slides. From histologically benign (Weiss scores 0–2) tumours, three 1-mm cores, and from histologically malignant (Weiss scores 3–9) tumours, six cores were obtained using a semiautomatic TMA instrument (Beecher Instruments, Silver Spring, MD, USA) with two cores also taken from the normal adrenal cortex.

4.5. Molecular analysis of tumours

4.5.1. Immunohistochemistry and scoring

Immunohistochemistry (IHC). Sections from TMA blocks 4-µm-thick were deparaffinised in xylene and rehydrated through a graded alcohol series. Table 8 summarises the antibody clones, dilutions, manufacturers, pretreatment and

endogenous peroxidase-blocking procedures. Slides were counterstained with Mayer's Hematoxylin (Lillie's Modification; Dako).

Two pathologists independently completed the **scoring** in studies III and IV. Any discrepancies between the evaluations were discussed until consensus was reached. Scorings of Ki-67 and cyclin D1 were conducted using the ImmunoRatio image analysis software, described below. The details of scorings used appear in Table 8.

4.5.2. Amplicon-based hot spot panel sequencing

DNA was extracted from 10 FFPE samples after deparaffinisation using the Maxwell® LEV Blood DNA Kit (Promega Corporation, Madison WI, USA) according to the manufacturer's instructions. The tumour content of each sample was evaluated from HE-stained slides by an experienced pathologist.

DNA was subjected to library preparation with the Ion AmpliSeq Cancer Hotspot Panel version 2, designed to target 2800 COSMIC mutations from 50 oncogenes and tumour suppressor genes, and then sequenced on the Ion Torrent PGMTM System (Thermo Fisher Scientific). The panel covers 15 and 11 hotspot regions in *IDH1* including the R132 and *IDH2* genes, respectively. Library preparation, template preparation and the sequencing were completed according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). Data analysis was carried out with the Torrent Suite Software version 4.0. After trimming and alignment to the hg19 human reference genome, sequence variants were detected using the VariantCaller version 4.0. The Ion Reporter software version 4.0 was used to filter out noncoding and polymorphic variants.

4.5.3. Hybridisation capture-based targeted sequencing

To prepare the paired-end libraries, 7000 to 32 000 ng of DNA extracted from FFPE blocks (n = 20) were used. For fresh-frozen samples (n = 16), 2300 to 14 300 ng of DNA was used as the input (ThruPlex DNA-Seq Kit, Rubicon Genomics). Target enrichment was performed using the SeqCap EZ Comprehensive Cancer panel (Roche Nimblegen). This panel covers 9 exons and the 5' untranslated region (UTR) of the *IDH1* gene, including the mutation sites of the Hot Spot Panel version 2. The enriched libraries were sequenced using the HiSeq2000 instrument with a paired-end 100-bp read length. Sequence analysis and variant calling were performed by an in-house bioinformatics pipeline (VCP, Variant Calling PipelineVCP (Sulonen et al. 2011)). In addition to the variant-calling algorithms, *IDH1* mutation hotspot loci were also visually inspected from the .bam files using the Integrative Genome Viewer (IGV (Robinson et al. 2011)). Library preparation, sequencing, variant calling and data analysis on this experiment were performed by the FIMM Sequencing Unit, HiLIFE, University of Helsinki, Finland.

Table 8. Details of the immunohistochemistry and scoring.

Antibody	Study	Clone	Company	Dilution	Detection	Pretreatment	Endogenous peroxidase blocking	Scoring used
Ki-67	II, III, IV	Mouse MIB-1	Dako, Glostrup, Denmark	1:200	Dako REAL EnVision / HRP, DAB + Chromogen	Tris-EDTA	0.3% Dako REAL Peroxidase-Blocking Solution	Nuclear staining: percentage of positive tumour cells, precision of 1%
c-myc	III	Mouse 9E10	Santa Cruz, UK	1:400	Dako REAL EnVision / HRP, DAB + Chromogen	Tris-HCL	0.3% Dako REAL Peroxidase-Blocking Solution	Nuclear staining: percentage of positive tumour cells 0 = 0, 1 < 30%, 2 = 30–50%, 3 = 50–80%, 4 > 80% Cytoplasmic staining: intensity 0 = none, 1 = weak, 2 = moderate, 3 = strong
p27	III	Mouse IgG_1 Kip1 aa. 1-197	Transduction Laboratories, KY, USA	1:100	Vectastain ABC kit	citrate	0.5% H2O2	Nuclear staining: percentage of positive tumour cells, precision of 10%
Cyclin D1	III	Rabbit SP4-R	Roche, Tucson, AZ, USA	ready to use	ultraView DAB	standard CC1	according to manufacturer	Nuclear staining: percentage of positive tumour cells, precision of 1%
Cyclin E	III	Mouse IgG_{2B} 713573	R&D Systems, MN, USA	1:600	Dako REAL EnVision / HRP, DAB + Chromogen	Tris-EDTA	0.3% Dako REAL Peroxidase-Blocking Solution	Nuclear staining: percentage of positive tumour cells, precision of 10%
IDH1 R132H	IV	Mouse H09	Dianova, Hamburg, Germany	1:20	Dako REAL EnVision / HRP, DAB + Chromogen	Tris-EDTA	0.3% Dako REAL Peroxidase-Blocking Solution	Cytoplasmic staining: intensity 0 = none, 1 = weak, 2 = moderate, 3 = strong

4.6. Digital pathology

Assessing the percentage of positive tumour cell nuclei in Ki-67 and cyclin D1 stainings was conducted using an internet-based open-source image analysis software called ImmunoRatio, which can be used in the quantitative assessment of any nuclei markers (Tuominen et al. 2010). Image capture was performed using a Nikon Eclipse 80i light microscope (40x objective) connected to a Digital Sight DS-5M (Nikon) digital camera and NIS-Elements F 3.0 image capture software. From each core biopsy, one digital image (JPEG format, Resolution 1280 x 960) was captured from the area of highest positivity, covering 50% of the core biopsy. With ImmunoRatio's Advanced Mode setting, threshold values for hematoxylin (0) and 3,3'-diaminobenzidine (DAB) (-10) were adjusted, with no correction equation. A blank-field image was taken to balance uneven illuminations in the final digital images. Image analysis settings remained identical after light exposure (manual exposure, 10 ms, gain 1x), and the thresholds were considered good. Figure 11 shows two examples of the original image and the pseudoimage created using ImmunoRatio, from which the percentage of positive nuclei was calculated. From all images available for each tumour, the highest count was recorded.

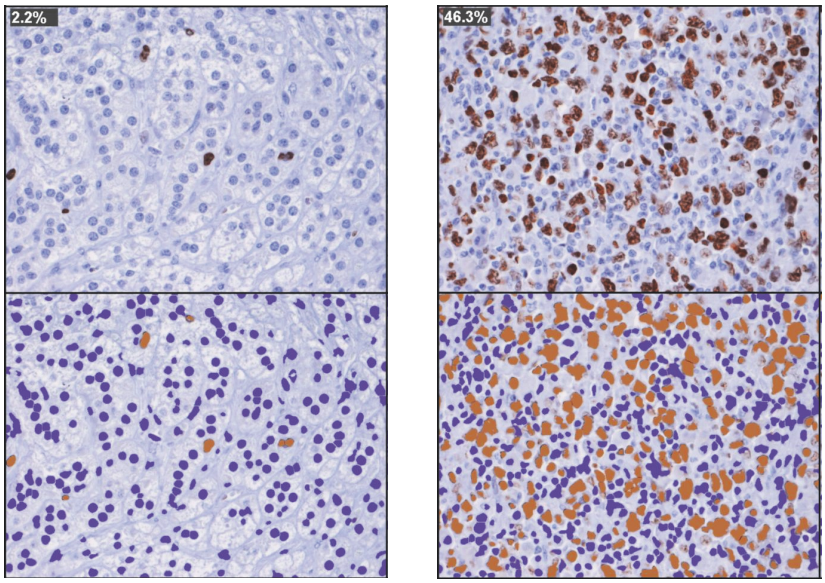

Figure 11. Two examples of defining proliferation index using ImmunoRatio. ImmunoRatio uses the original immunostaining (the upper image) to create a pseudoimage (the lower image), from which the precise proliferation index is calculated.

4.7. Statistical analysis

In study I, the Mann–Whitney U-test was used to assess the significance of the difference in the mean rank of unenhanced attenuation values (HU) for tumours with or without the individual Weiss criterion. The significance of the differences in the unenhanced CT attenuation value (HU) between the three tumour groups (lipid-rich adenomas, lipid-poor adenomas and malignant tumours) was assessed using the Kruskal–Wallis analysis of variance (ANOVA) after adjusting for multiple comparisons in pairwise comparisons. The correlation of the total Weiss score as well as the eosinophilic cell count with the unenhanced CT attenuation value (HU) was assessed with the Spearman's rank correlation test. We considered $p < 0.05$ as statistically significant. The statistical analyses were performed using SPSS version 20.0 software (IBM SPSS Statistics, SPSS Inc., Chicago, IL, USA).

In studies II through IV, results are reported as the mean, median and range, or as the number of patients and proportion of patients. The Mann–Whitney U-test served to analyse differences between patient groups for continuous and ordinal variables. The Fisher's exact test analysed differences in dichotomous variables. The Spearman's rho correlation coefficient was calculated between different scores and the metastatic status. Survival rates were estimated using the Kaplan–Meier method and the log-rank test compared survival curves. We considered $p < 0.05$ as statistically significant relying upon two-tail tests. Statistical calculations were performed using SPSS version 20 (IBM, New York, NY, USA) or with Statexact version 4 (Cytel Inc., Cambridge, MA, USA) in study II, with SPSS version 22 (IBM, New York, NY) in study III and using SPSS version 24 (IBM, New York, NY, USA) in study IV.

In study II, the Clopper–Pearson 95% confidence intervals (CI) were calculated for the sensitivity and specificity values. Differences in specificities between different scores were tested using the nonparametric paired McNemar's test. Logistic regression and receiver-operating characteristic (ROC) analyses served to model the new score developed here. Logistic regression with backward stepping was used to identify the most influential variables, and ROC analysis to determine the optimal cutoff value by maximising Youden index.

4.8. Ethical approvals

This study was approved by the local ethics committee (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013) and the National Supervisory Authority of Health and Welfare (TEO Dnro 10041/06.01.03.01/2012).

5 RESULTS

5.1. Unenhanced CT attenuation value to assess adrenal tumours (study I)

The total Weiss score correlated with the unenhanced CT attenuation value (HU) ($r_s = 0.582$, $p < 0.001$). The higher the score, the higher the CT attenuation value.

The eosinophilic cell count was unevenly distributed across the tumour material: most tumours contained either less than 30% or more than 80% eosinophilic cells. The unenhanced CT attenuation value correlated with eosinophilia ($r_s = 0.750$, $p < 0.001$). However, some tumours showed a substantial deviation from the regression line.

To compare the lipid-rich adenomas, lipid-poor eosinophilic adenomas and malignant tumours, we grouped tumours into three groups according to the lipid content and the level of malignancy. Here, we used one of the Weiss criteria (<25% lipid-rich cells) to define a lipid-poor eosinophilic adenoma. The percentage of eosinophilic cells and unenhanced CT attenuation value according to the tumour groups appear in Table 9. In the Kruskal–Wallis ANOVA, the distribution of unenhanced CT attenuation value was significantly different across groups ($p < 0.001$). In the pairwise comparisons adjusted for multiple comparisons, lipid-rich tumours (group 1) had a significantly lower unenhanced CT attenuation value than lipid-poor tumours (group 2) ($p < 0.001$) or malignant tumours (group 3) ($p < 0.001$). However, we found no difference between lipid-poor adenomas and malignant tumours ($p = 1.000$).

None of the malignant tumours had a CT attenuation value under 22 HUs.

Table 9. Percentage of eosinophilic cells and unenhanced CT attenuation value (Hounsfield units, HU) according to tumour groups. Comparisons are based on Kruskal–Wallis ANOVA. Groups 1 and 2 include Weiss score 0–2 tumours and group 3 Weiss score 3–9 tumours.

		Group 1 lipid-rich	Group 2 lipid-poor	Group 3 malignant
Number of tumours		54	14	11
Eosinophilia %	range	0–50	70–100	80–100
	median	12.4	82.9	90
Hounsfield units	range	-10–40	21–43	22–44
	median	7	30	33
	mean rank	28.7	63.2	65.8
Comparison between groups	1 vs 2	$p < 0.001$		
	1 vs 3	$p < 0.001$		
	2 vs 3	$p = 1.000$		

5.2. Optimising histological scoring systems: the Helsinki Score (study II)

In assessing the diagnostic power of scoring systems, tumours were defined as either local or metastatic. Table 10 shows the distribution of the Weiss score in this material. **The Weiss score** was sensitive in distinguishing all metastatic carcinomas. However, a number of local tumours were falsely diagnosed as malignant. In diagnosing a metastatic ACC, the sensitivity of the Weiss system was 100% (CI 76.8–100%), whilst the specificity reached 90.2% (CI 84.6–94.3%; see Table 12).

The proliferation index (PI) of the tumours was assessed using the Ki-67 stainings relying on the ImmunoRatio image analysis software. Table 10 shows the range, mean, median and interquartile range (IQR) of the PI in this material. A significant difference in PI between local and metastatic tumours ($p < 0.01$) was identified. A cutoff value of 4% was considered optimal. Using this cutoff value, PI diagnosed metastatic carcinoma with a 71.4% sensitivity (CI 42.0–90.4%) and 96.9% specificity (CI 92.4–98.8%; see Table 12).

Logistic regression with backward stepping was used to identify the most influential variables for the nine Weiss criteria plus the PI in this tumour material. This led us to a scoring model, called the Helsinki score: 3.28 x mitotic rate + 4.42 x necrosis + 1.02. This was simplified to 3 x mitotic rate + 5 x presence of necrosis + proliferation index (see Figure 12). ROC analysis was used to determine the optimal cutoff value.

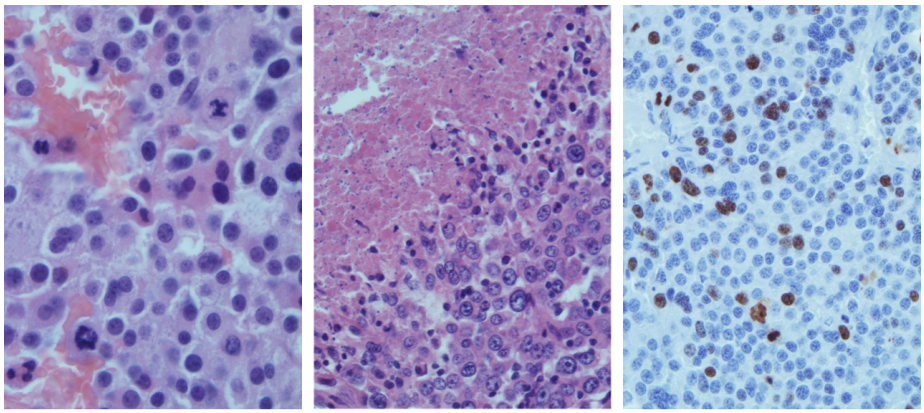

Figure 12. The Helsinki score = (3 x mitotic rate > 5/50 HPF + 5 x necrosis + proliferation %), illustrated by examples of each of these three parameters.

Table 10. Distribution of tumours according to the Weiss score, the Helsinki score and the proliferation in local versus metastatic tumours.

		All	Local	Metastatic
Number of tumours		177	163	14
Weiss score	0–2	147	147	0
	3–9	30	16	14
Helsinki score	<8.5	162	162	0
	≥8.5	15	1	14
Proliferation %	range	0–54.2	0–8.0	0.9–57.2
	mean	2.3	1.0	16.7
	median	0.8	0.7	7.0
	IQR		1.0	22.5

IQR, interquartile range

The Helsinki score, with a cutoff value of 8.5, diagnosed a metastatic ACC with a sensitivity of 100% (CI 76.81–100%) and a specificity of 99.4% (CI 96.6–100%). The comparison of the Helsinki score to the Weiss score in this series appears in Tables 11 and 12. Only one tumour earned a false-positive result using the new scoring system. This specific tumour was considered malignant with high scores

according to both scoring systems, with a Weiss score of 9 and a Helsinki score of 11.3. The tumour was large (28 cm) and locally invasive, but radically excised. This patient received mitotane treatment following surgery, and was alive at the end of the follow-up period 8 years after surgery.

Table 11. Comparison of the Weiss score and the Helsinki score.

		Weiss score		
		0–2	**3–9**	
Helsinki score	**<8.5**	147	15	162
	≥**8.5**	0	15	15
		147	30	

Table 12. Sensitivity and specificity in diagnosing metastatic adrenocortical carcinoma (ACC) using the Weiss score, the proliferation index (PI) and the Helsinki score in this tumour series.

	Sensitivity	**Specificity**
Helsinki score <8.5 vs ≥8.5	100% CI 76.8–100%	99.4% CI 96.6–100%
Weiss score 0–2 vs 3–9	100% CI 76.8–100%	90.2% CI 84.6–94.3%
Proliferation index ≤4% vs <4%	71.4% CI 42.0–90.4%	96.9 % CI 92.4–98.8%

CI, confidence interval

A Helsinki score higher than 8.5 correlated with survival (see Figure 13). Based on their Helsinki score, tumours were categorised into three groups: 1) benign tumours with a Helsinki score of 0 to 8.5; 2) malignant tumuors with a Helsinki score of 8.5 to 17; and 3) malignant tumours with a Helsinki score of >17. Differences in survival amongst these three groups were significant: group 1 vs. group 2, $p < 0.01$; group 1 vs. group 3, $p < 0.01$; group 2 vs. group 3, $p = 0.010$.

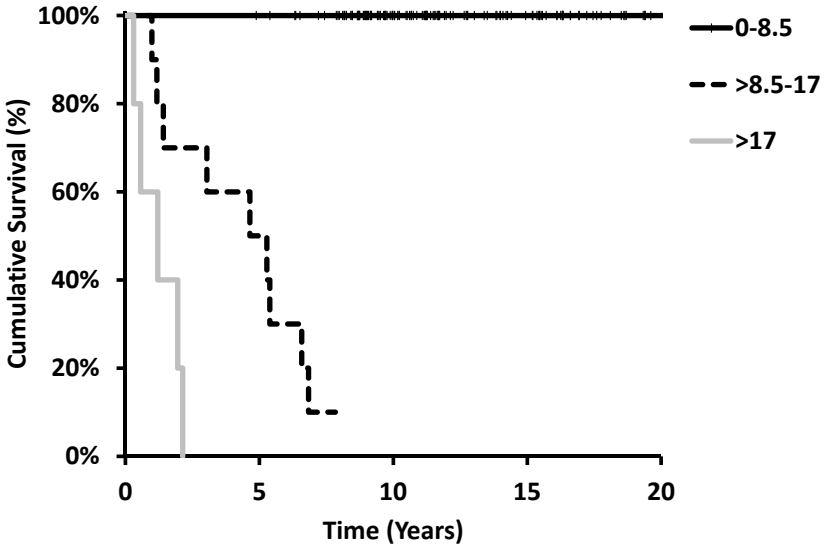

Figure 13. Survival according to the Helsinki score.

5.3. Molecular markers of malignancy

5.3.1. Transition of c-myc expression from the nucleus to cytoplasm indicative of malignant transformation (study III)

ACTs show both **nuclear and cytoplasmic staining** of c-myc. Table 13 shows how the staining **differed between local and metastatic** tumours. In benign adenomas, the cytoplasm did not stain or stained weakly (0–1), whereas nuclear staining was moderate to strong (3–4). This was also the case in the normal adrenal cortex (data not shown). In metastatic carcinomas, the cytoplasmic staining was typically strong and nuclear staining appeared weaker than in adenomas. Figure 14 shows the typical staining patterns of c-myc in adenomas and carcinomas. The difference both in the cytoplasmic and in the nuclear staining of c-myc between local and metastatic tumours was significant ($p < 0.001$ and $p = 0.004$).

Table 13. Distribution of immunohistochemical (IHC) scores in studies III and IV. The Fisher's exact test compared IHC scores between local and metastatic tumours.

		All	Local	Metastatic	p value
Number of tumours		197	183	14	
c-myc					
cytoplasmic score 0–3	range	0–3	0–3	1–3	<0.001
	mean	1.4	1.3	2.6	
	median	1.0	1.0	3.0	
nuclear score 0–4	range	0–4	0–4	0–4	0.011
	mean	3.1	3.2	2.1	
	median	4.0	4.0	1.5	
cytoplasmic-to-nuclear ratio	≤0.75	142	141	1	<0.001
	>0.75	50	37	13	
p27 %	range	0–100	0–100	20–100	0.307
	mean	53.8	53.0	63.6	
	median	70.0	70.0	75.0	
Cyclin E %	range	0–80	0–80	0–60	0.001
	mean	8.7	7.7	21.4	
	median	5.0	5.0	15.0	
Cyclin D1 %	range	0–90	0–90	0–50	0.227
	mean	12.6	12.1	18.6	
	median	5.0	9.0	16.5	
IDH1 R132H score 0-3	range	0–3	0–3	0–2	0.699
	mean	0.4	0.4	0.3	
	median	0	0	0	

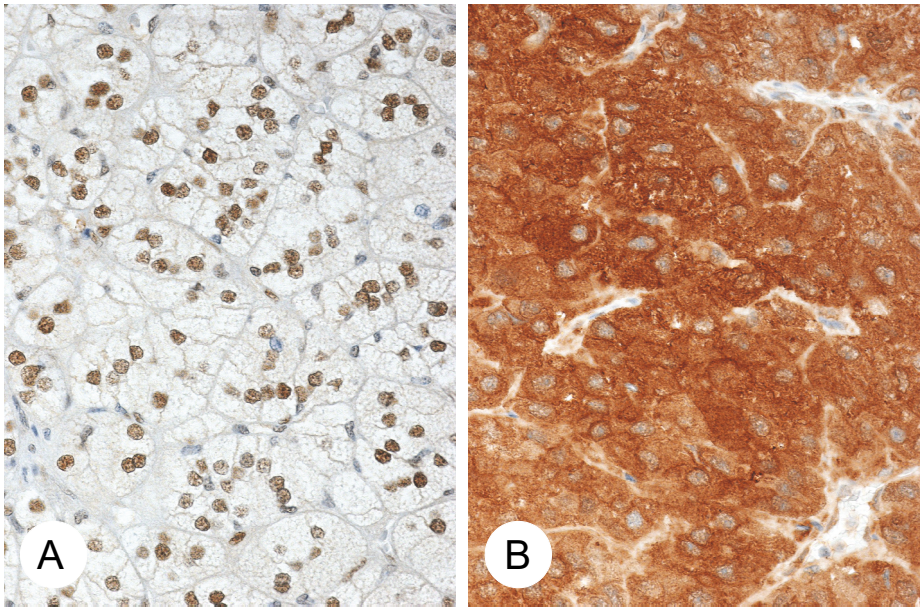

Figure 14. Typical staining pattern of c-myc in an adenoma and a carcinoma. A) An adenoma shows strong nuclear c-myc staining and no cytoplasmic staining. B) A carcinoma shows strong cytoplasmic c-myc staining and weak to no nuclear staining. Images: 400x objective.

The optimal cutoff value for cytoplasmic and nuclear c-myc staining distinguishing local tumours from metastatic tumours was 3 ($p < 0.001$ and $p = 0.004$). To further investigate the importance of the localisation of the c-myc protein, we calculated the **ratio of cytoplasmic to nuclear** c-myc for each tumour. In order to avoid dividing by zero, we increased each score of nuclear c-myc by one. The difference in this new parameter between local and metastatic tumours was significant ($p < 0.001$). The optimal cutoff value for the cytoplasmic-to-nuclear c-myc ratio distinguishing local tumours from metastatic tumours was 0.75 ($p < 0.001$).

Cytoplasmic c-myc staining correlated with the total Weiss score for the tumour ($R_s = 0.531$, $p < 0.001$) and with the Helsinki score ($R_s = 0.283$, $p < 0.001$). The cytoplasmic-to-nuclear c-myc ratio also correlated with the Weiss score ($R_s = 0.398$, $p < 0.001$) and the Helsinki score ($R_s = 0.193$, $p = 0.008$). Yet, nuclear c-myc staining did not correlate with the Weiss ($p = 0.102$) nor the Helsinki score ($p = 0.133$).

Kaplan–Meier **survival** analysis identified an association between both cytoplasmic and nuclear staining and survival (log-rank test, $p < 0.001$ and $p = 0.008$).

C-myc–related molecules cyclin D1, p27 and cyclin E were also studied to investigate the correlation of c-myc expression to molecules associated with the signaling pathway. The distributions of nuclear cyclin D1, p27 and cyclin E IHC

stainings appear in Table 13. No significant difference in the nuclear stainings of cyclin D1 or p27 between local and metastatic tumours was found (p = 0.227 and p = 0.307), but the difference in nuclear cyclin E staining was significant using the optimal cutoff value of 35 (p = 0.001). The correlation of c-myc, cyclin D1, p27 and cyclin E expressions to each other appear in Table 14.

Table14. Correlations between the c-myc, cyclin D1, p27 and cyclin E expressions in adrenocortical tumours (ACTs). The Spearman's rho correlation coefficient was calculated between different scores. Statistically significant correlations are on grey background.

	c-myc		Cyclin D1	p27
	nuclear	cytoplasmic		
Cyclin D1	R_s = 0.148 p = 0.040	R_s = 0.260 p < 0.001		
p27	R_s = 0.106 p = 0.144	R_s = 0.303 p < 0.001	R_s = 0.388 p < 0.001	
Cyclin E	R_s = -0.062 p = 0.395	R_s = 0.110 p = 0.130	R_s = 0.197 p = 0.006	R_s = 0.143 p = 0.046

5.3.2. Positive mutation-specific IDH1 R132H immunostaining indicates better prognosis amongst carcinoma patients (study IV)

The IDH1 R132H IHC staining appeared as a granular cytoplasmic positivity in ACTs. Similar staining appeared in the normal adrenal cortex. The intensity of the staining varied and was scored on a scale from 0 to 3.

The IDH1 R132H staining appeared in both benign and malignant tumours, and negative vs. positive staining **did not distinguish between local and metastatic** disease (p = 0.359). Table 14. summarises the distribution of IDH1 R132H staining in this material.

The aldosterone secretion of the tumour correlated with a weak IDH1 R132H staining (R^2 = -0.163, p = 0.023), whereas cortisol secretion correlated with a strong IDH1 R132H staining (R^2 = 0.243, p = 0.001). We found no correlation between the IDH1 R132H staining and the testosterone secretion (R^2 = 0.071, p = 0.326) or hormonal inactivity (R^2 = -0.132, p = 0.066).

To examine the possible **prognostic significance** of the IDH1 R132H staining in ACCs, all 33 tumours with a Weiss score of 3 to 9, defined as potentially malignant according to the WHO classification, were selected. Patients with a positive IDH1 R132H staining survived longer than patients with negative staining (p = 0.031).

To identify known **mutations of IDH1 at Arg132**, 10 tumours with Weiss scores of 3 to 9 were selected, 5 of which exhibited a strong positive and 5 of which exhibited a negative IDH1 R132H IHC staining. Using amplicon-based hotspot

panel sequencing, with a sensitivity of 2%, we identified no mutations in the *IDH1* or *IDH2* genes.

For the **IDH1 gene sequencing** from the formalin-fixed paraffin-embedded tumour material, 10 additional tumours were selected: 5 tumours with Weiss scores of 0 to 2 with positive IDH1 R132H IHC staining and 5 tumours with Weiss scores of 3 to 9 with low positivity. In total, 20 tumours were analysed. Using hybridisation capture–based targeted sequencing, no informative sequencing data were received. Initial sequencing provided the mean target coverage of the sequencing depth after removing duplicates. Sequencing delivered depths of 115 to 505x; yet, after PCR duplicate removal, the informative depth diminished to 2x to 13x. Evidently, the samples contained sufficient DNA molecules (mass), but the mass was heavily cross-linked and/or fragmented, thereby preventing successful library preparation. Such DNA behaves seemingly well in PCR amplicon-based methods, but lacks a true somatic resolution given the limited number of accessible DNA template molecules.

Amongst the 20 sequenced formalin-fixed paraffin-embedded tumours, fresh-frozen tumour material was available from 16 tumours. Using the same protocol for the hybridisation capture–based targeted sequencing, depths ranging from 100 to 500x after PCR duplicate removal were achieved, enabling the performance of somatic mutation calling. However, no known hotspot mutation nor any novel variants in the *IDH1* gene nor the UTRs were found.

6 DISCUSSION

6.1. Rising incidence due to incidentalomas

Characterising adrenal tumours has become an important clinical issue given the widespread use of radiological imaging and, thus, the increased incidence of clinically inapparent lesions, or 'incidentalomas'. In addition, adrenal tumours are detected more often in ageing Western populations, since their incidence increases with age. Most adrenal tumours are benign cortical adenomas detected either through symptoms caused by excess hormone secretion or as incidental findings in radiological imaging. Adrenocortical carcinomas (ACCs) remain rare with a stable incidence of 1 to 2 cases per million population per year (Lloyd et al. 2017a). Whilst carcinomas are primarily detected through symptoms related to hormone excess or mass effect, incidentalomas may also prove to be carcinomas. In the preoperative assessment of adrenal tumours, phaeochromocytoma and metastasis to the adrenal gland must also be considered. Thus, patient history, clinical examination, biochemical measurements and radiological imaging all contribute to the initial evaluation (Fassnacht et al. 2016).

6.2. Unenhanced CT: the benign and the indeterminate

The primary modality for the radiological assessment of adrenal tumours is unenhanced CT. Characteristics such as a large size, uneven border, heterogenous consistency and irregular calcifications suggest malignancy as expected. In addition, a high CT attenuation value, defined by the Hounsfield units, associates with malignancy. Lipids are radiotranslucent and, since most benign cortical adenomas are lipid-rich, a low attenuation value indicates a benign adenoma. In general guidelines, including the 'Clinical Practice Guidelines for the Management of Adrenal Incidentalomas' by the European Society of Endocrinology, the threshold is set at ≤10 HUs (Fassnacht et al. 2016). Thus, if the tumour has benign features and an attenuation value ≤10 HUs, the tumour can be diagnosed as a benign adenoma and no further investigations are necessary. A tumour with any malignant features and/or attenuation >10 HUs is considered indeterminate and must be further examined.

Indeterminate tumours include both malignant tumours and benign adenomas containing fewer lipids, also referred to in radiological terms as lipid-poor adenomas. However, only one group has an established correlation between the lipid content and the unenhanced CT attenuation value in adrenocortical tumours (ACTs)

(Korobkin et al. 1996). Their material was small, consisting of 20 adenomas and only a few lipid-poor adenomas. We confirmed in our study of 79 clinically verified ACTs that the proportion of eosinophilic lipid-poor cells correlates with the CT attenuation value or HUs. However, a large proportion of tumours >10 HUs were actually lipid-rich, whereby other factors contributed to the CT attenuation than lipid content alone. Furthermore, and more importantly from the patient's point of view, all carcinomas had attenuation values >21 HUs. Thus, according to our material, the threshold for an indeterminate tumour could be raised from 10 to 20 HUs.

Thus far, the CT attenuation threshold for indeterminate tumours has remained >10 HUs, achieving a maximum sensitivity in discerning a malignancy. In a meta-analysis by ESE consisting of 18 adrenal tumour imaging studies, the imaging modalities and the background of the patients varied (Fassnacht et al. 2016). All unenhanced CT studies used the same attenuation threshold of >10 HUs for indeterminate tumours. The sensitivity in diagnosing a malignant tumour amongst pure incidentalomas was determined in only two studies, reaching 100% and 95%. Sensitivity dropped to 93% amongst patients with a history of extra-adrenal malignancies in five studies. However, no other attenuation thresholds were used in these studies and not all diagnoses were histologically verified. Since unenhanced CT is the first imaging modality used to assess the nature of adrenal tumours, dividing tumours accurately into benign and indeterminate is especially important to rule out as many patients as possible from further imaging examinations or follow-up. These examinations expose the patient to irradiation and cause mental stress. All medical procedures also carry a financial burden to the healthcare system. As the incidence of incidentalomas increases, we should consider raising the CT attenuation threshold for indeterminate tumours, at least amongst patients with no history of malignancy. Furthermore, multicentre studies involving extensive material comparing the CT attenuation value of adrenal tumours with an accurately defined histological diagnosis and outcome would likely benefit patients and the healthcare system as a whole.

6.3. Characterising a malignancy: striving for accuracy

Metastasis represents a definitive sign of malignancy in ACTs as well as the primary cause of a fatal outcome; thus far, metastatic disease cannot be cured. In nonmetastatic tumours, the malignant potential must be assessed histologically. Since many histological parameters associate with a malignancy, various histological scoring systems combining various parameters have been proposed as predicting malignancy as accurately as possible. The most widely used system is the Weiss system (Weiss 1984), which has served as the gold standard globally for several decades. Thus, most pathologists are familiar with the nine histological criteria in

the Weiss system. This system is sensitive in diagnosing ACCs, and the score also correlates with prognosis. However, pathologists are also painstakingly aware of the faults in the Weiss system: some criteria remain difficult to assess, resulting in interobserver variation (Aubert et al. 2002; Tissier et al. 2012). Some tumours with borderline scores never recur or metastasise, thereby leading to questions regarding the specificity of the system. Furthermore, the Weiss system does not apply to histological subtypes or children's tumours (de Krijger et al. 2012; Dehner et al. 2009).

Proliferation using Ki-67 IHC staining has been used as an additional tool to assess malignancy and prognosis in ACTs (Beuschlein et al. 2015; Morimoto et al. 2008; Soon et al. 2009; Terzolo et al. 2001; Wachenfeld et al. 2001). Where the Weiss system is sensitive in diagnosing ACCs, proliferation has proved specific in diagnosing a malignancy. Some ACCs present with a low proliferation and are not diagnosed as carcinomas even with a fairly low cutoff value.

Thus, we proposed a new scoring system to predict the metastatic potential of adult ACTs. The Helsinki score combines two of the most objective histological Weiss criteria with the proliferation index, achieving a reliable and powerful diagnostic system. The Helsinki score, calculated as 3 x mitotic activity >5/50 HPF + 5 x presence of necrosis + proliferation %, uses a cutoff value of ≥8.5 to define a carcinoma. The Helsinki score also correlates with survival. The prognostic significance of the Helsinki score was validated by Duregon et al. (2017) in a study on 225 ACCs, where the Helsinki score outperformed the Weiss score, mitotic index, proliferation and tumour stage as prognostic parameters. The extensive material also included oncocytic and myxoid tumours, verifying the prognostic value of the Helsinki score in these histological subtypes. Another study of 43 oncocytic tumours compared the Helsinki score with the Weiss score, the Lin–Weiss–Bisceglia score and reticulin algorithm in diagnosing a malignancy (Renaudin et al. 2018). The Helsinki score was by far the most powerful diagnostic system for oncocytic tumours.

6.4. C-myc: the transition from nucleus to cytoplasm associates with malignancy

The overexpression of the transcription factor c-myc has been associated with multiple cancers (Bai et al. 1994; Calcagno et al. 2009; Pietilainen et al. 1995), following the detection of its role in Burkitt's lymphoma (Dalla–Favera et al. 1982). Yet, the connection between c-myc expression and adrenocortical neoplasia has received very little attention. Only one study has described the IHC expression of c-myc in ACTs. In that study, c-myc expressed in the nuclei of all 15 tumours. In carcinomas, c-myc staining also occurred in the cytoplasm. (Suzuki et al. 1992). Furthermore, even the underexpression of c-myc in adrenocortical neoplasia has

been suggested based on the pathway analysis of the gene expression microarray- and comparative genome hybridisation studies (Szabo et al. 2010; 2011). However, one pathway leading to the c-myc expression, the Wnt/ß-catenin pathway, has been associated with adrenocortical neoplasia in various studies (Berthon et al. 2010; Bonnet et al. 2011; Durand et al. 2011; Gaujoux et al. 2011; Parviainen et al. 2013; Ragazzon et al. 2010; Tissier et al. 2005).

We showed that ACTs express c-myc. C-myc appears in the nuclei of benign adenomas as well as in the normal adrenal cortex. In carcinomas, c-myc appears in the cytoplasm and clears from the nucleus. Cytoplasmic c-myc has also been found in other neoplasias (Bai et al. 1994; Calcagno et al. 2009; Pietilainen et al. 1995; Ruzinova et al. 2010), thereby correlating with cancer aggressiveness. Conacci–Sorrell et al. (2010, 2014) rigorously studied the role of cytoplasmic c-myc. They presented a truncated, transcriptionally inactive cytoplasmic c-myc protein called myc-nick, which promotes cancer cell survival and augments cancer cell motility. Myc-nick is generated in response to metabolic and cytotoxic stress by calpain-mediated proteolysis under which conditions the full-length c-myc would cause cell death by apoptosis. In addition, Conacci–Sorrell et al. (2010, 2014) showed that myc-nick expresses in a wide range of tumours, hypothesising that it could represent the cytoplasmic c-myc immunostaining seen in some previous studies. The cytoplasmic c-myc in ACTs, however, does not appear to be myc-nick. The 9E10 antibody we used for IHC binds to the C-terminus of the c-myc protein, which truncates in the myc-nick protein. It is possible that the cytoplasmic c-myc seen in ACTs represents a full-length c-myc protein retained in the cytoplasm under stress conditions, awaiting processing to myc-nick similarly to processes at the invasive front in colon cancer (Conacci–Sorrell et al. 2014).

We also compared the c-myc expression with that of cell cycle–related proteins cyclin E, cyclin D1 and p27 to further examine the role of c-myc. Our nuclear staining of cyclin E was higher in metastatic than in local tumours, agreeing with a previous study (Tissier et al. 2004). However, neither cytoplasmic nor nuclear c-myc staining correlated with cyclin E. Thus, c-myc and cyclin E appear to play independent roles in ACT development, suggesting that cyclin E may not be the target gene of c-myc in ACCs. Cyclin D1 showed a higher staining in metastatic tumours than in local tumours, but the difference was not significant. Cyclin D1 correlated with the cytoplasmic c-myc expression and to a lesser extent with nuclear c-myc, suggesting a common inducer for the expression of both proteins. The inducer could be the Wnt/ß-catenin pathway targeting both cyclin D1 and c-myc in colon carcinomas (He et al. 1998) and also associating with adrenocortical neoplasia.

The expression of the cell-cycle inhibitor protein 27 (p27 or cyclin-dependent kinase inhibitor 1B) associates with a good prognosis in several cancers (Migita et al. 2002; Nozoe et al. 2006). However, p27 plays a dual role in cancer, since elevated levels have been associated with a metastatic potential (Kouvaraki et al. 2002).

C-myc appears to inhibit the p27 expression. Our mean nuclear p27 staining was slightly higher in metastatic than in local tumours, although this difference was not significant; p27 correlated with cytoplasmic c-myc, but not with nuclear c-myc and correlated with cyclin E. C-myc does not appear to inhibit p27 expression in ACTs, although the correlation with cyclin E indicates a possible role in inhibiting the cell cycle–promoting function of overexpressed cyclin E.

Over all, the strong cytoplasmic c-myc and weak nuclear c-myc expression in ACTs associated with the metastatic potential and shorter survival. We hypothesise that the transition of c-myc from the nucleus to cytoplasm prevents c-myc from acting as a transcription factor and from inducing apoptosis. Further studies are necessary in order to uncover the mechanism of the c-myc function in ACTs.

6.5. Isocitrate dehydrogenase (IDH)

Oncogenic mutations of the metabolic enzyme IDH have been reported in several neoplasias (Amary et al. 2011; Borger et al. 2012; Mardis et al. 2009; Murugan et al. 2010), but have thus far remained unreported in ACTs. IDH mutations were first detected in gliomas, where these mutations indicate a distinctive pathogenesis and prognosis (Parsons et al. 2008). A specific R132H mutation to IDH1 accompanies approximately 80% of all IDH mutations in gliomas (Hartmann et al. 2009). To improve the diagnosis of gliomas, Capper et al. (2009) developed an antibody that binds to the mutated site of IDH1 R132H. This mutation-specific antibody can be used to detect the mutated protein from formalin-fixed paraffin-embedded tissues through IHC.

Another research group in Helsinki identified IHC positivity for IDH1 R132H in the normal adrenal cortex (oral communication), thereby increasing interest in the examination of IDH1 R132H expression in ACTs. We found granular cytoplasmic immunostaining for IDH1 R132H in the normal adrenal cortex and in ACTs to varying degrees. IDH1 R132H IHC did not distinguish between local and metastasised disease. However, a positive IDH1 R132H IHC correlated with a longer survival amongst ACC patients. In genetic analysis, we identified no known or novel IDH1 mutations in ACTs. The epitope binding IDH1 R132H antibody in ACTs remains unknown. Nevertheless, a significant difference in survival between immunopositive and immunonegative carcinomas indicates that the epitope carries some clinical relevance. This difference in survival may associate with the natural disease progression or the treatment response of different tumour subtypes. There may be a post-transscriptional modification of the IDH1 RNA or a conformational alteration of the protein. Furthermore, the binding of the specific IDH1 R132H antibody to ACTs indicates that new experimental therapies such as small molecule

inhibitors of mutated IDH or peptide vaccines (Friedrich et al. 2018; Golub et al. 2019) may benefit patients with metastatic ACCs.

6.6. Strengths and limitations

A major advantage of this study is its large cohort size and the comprehensive clinical and follow-up data on all patients. That is, no patients were lost to follow-up. One limitation is the small number of rare metastatic carcinomas in a small population.

In study I, the unenhanced CT attenuation value of tumours was compared to their histological diagnosis using the Weiss score. This represents a limitation since the Weiss score does not always reveal the true nature of a tumour. Some carcinomas in this material did not recur or metastasise. This limitation is shared with other studies, since it occurred in many studies where new diagnostic systems were developed. The diagnostic power has often been compared to that of the Weiss score, so that the diagnostic power has not improved. It would be better to compare any diagnostic parameter to the malignant outcome, whether defined by metastasis or disease-related death. The Helsinki score was developed by identifying the prognostically most relevant factors achieving diagnostic accuracy. The CT attenuation value of ACTs should also be compared to a more accurately defined diagnosis.

One strength in study II, in establishing the Helsinki score, lies in that the proliferation index was defined using computer-assisted image analysis. The quantitative assessment of IHC markers suffers from interobserver variability (Mengel et al. 2002; Papathomas et al. 2016), whilst the proliferation index using Ki-67 represents a significant factor in the Helsinki score. Still, interlaboratory variation remains a risk in assessing proliferation. The use of the proliferation index can be justified given its considerable role in determining ACC prognosis as well as the fact that it has enjoyed success in grading neuroendocrine tumours (Klimstra et al. 2019).

6.7. Future prospects

The findings in this thesis highlight the need for expertise in diagnosing ACCs and in determining the proper treatment strategy for a tumour patient. With a rare disease like ACC, patients should be referred to a specialised unit where all members of the interdisciplinary medical team are dedicated to finding the best possible individualised care for a patient. New diagnostic methods using sophisticated technology such as liquid biopsy are currently being studied. In addition, new therapeutic methods emerge often related to specific molecular markers associated

with a tumour (Crona et al. 2018). Thus, Helsinki score version 2.0 should be developed. This could integrate aspects included in version 1.0 as well as molecular and possibly imaging data.

The diagnosis of ACTs is based on histopathology, even with recent advances in molecular pathology. All further investigations of the tumour sample are decided upon by the pathologist. Therefore, all ACC diagnoses should be confirmed by an experienced endocrine pathologist. In future, this could be achieved through digital consultation, where slides are scanned and can be assessed anywhere in the world. More broadly, the medical team including radiologists, endocrinologists, surgeons, oncologists and medical geneticists must keep abreast with new developments to ensure high-quality diagnostics and management of ACC.

7 CONCLUSIONS

The main conclusions from the studies presented here are as follows:

1. The proportion of eosinophilic lipid-poor cells in ACTs correlates with the CT attenuation value by HUs, although CT attenuation cannot distinguish between lipid-poor adenomas and carcinomas. All carcinomas exhibited attenuation values >21 HUs. Thus, according to this material, the threshold for an indeterminate tumour could be increased from 10 to 20 HUs, at least amongst patients with no history of malignancy.

2. The Helsinki score, relying on the mitotic frequency, the proliferation index as well as the presence of necrosis, accurately predicts the metastatic potential of ACTs, outperforming the diagnostic power of the Weiss scoring system.

3. A strong cytoplasmic and weak nuclear c-myc expression in ACTs associate with malignancy and a shorter survival, suggesting that the transition of c-myc from the nucleus to cytoplasm may represent a pathogenic step in adrenocortical carcinogenesis.

4. Amongst ACCs, IDH1 R132H immunopositivity correlates with a better prognosis, although immunopositivity does not appear to associate with mutations to the IDH1 gene.

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